University of Alberta

Maximizing the Production of *Gamma*-Linolenic Acid in *Mortierella ramanniana* var. *ramanniana* as a Function of pH, Temperature, Carbon Source, Nitrogen Source, Metal Ions, and Oil Supplementation

by

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of the requirements for the degree of **Master of Science**

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List of Abbreviations

ALA	*alpha*-linolenic acid
ARA	arachidonic acid
CMB	cornmeal infusion broth
DYB	dextrose-yeast broth
EFA	essential fatty acid
FYB	fructose-yeast broth
GLA	*gamma*-linolenic acid
GYB	glycerol-yeast broth
LA	linoleic acid
MEYB	malt extract-yeast broth
OA	oleic acid
PA	palmitic acid
PDA	potato-dextrose agar
PDB	potato-dextrose broth
SA	stearic acid
SAYB	sodium acetate-yeast broth
SYB	sucrose-yeast broth

Chapter 1

Implications for the Use of *Mortierella* Fungi in the Industrial Production of Essential Fatty Acids

Introduction:

As North Americans become more conscious of the importance of good nutrition, the public has been bombarded with information regarding 'good' fats and 'bad' fats. The body of research concerning the effects of fats on human health is heavily reported in academic literature. It is now common knowledge that the 'bad' fats include saturated and *trans* fats while the 'good' fats include the *omega*-3 (ω-3) and *omega*-6 (ω-6) fatty acids. This latter group of fatty acids include *gamma*-linolenic acid (GLA) and linoleic acid (LA) both of which are essential fatty acids (EFAs).

Less attention has been focused on the sources of important fats, one source being oleaginous or "oil-bearing" organisms. Each oleaginous organism produces oil with a specific fatty acid profile. For example, the fatty acid profile produced by an organism to be used in production of cocoa butter substitutes will be different from the target profile of one that will be used in arachidonic acid (ARA) production. The specific organisms used in these studies will also differ depending on their fatty acid profiles. This paper focuses on the production of EFAs in *Mortierella* fungi.

Lipids:

Lipids play significant roles as structural as well as storage devices and are also important metabolic intermediates in oil-bearing organisms (1). Lipids are accumulated as lipid bodies in almost all eukaryotic organisms at some point during their life cycle. In yeast, for example, it has been observed that lipid accumulation occurs when the organism is under conditions of stress (e.g., when there is a high carbon to nitrogen ratio in the growth medium) (2).

The major component of lipids produced in oil-bearing organisms are triacylglycerol (TAG) molecules. TAG molecules consist of three fatty acids attached to a glycerol backbone, as shown in Figure 1-1. TAG and fatty acid compositions can vary both within and between organisms. Furthermore, research has shown that the fatty acid profiles of some organisms can be influenced by their external environments.

The position of fatty acids within a TAG molecule determines its bioavailability within the human body. Prior to utilization within the body, the fatty acids must first be freed from the glycerol backbone. This is accomplished by the action of digestive enzymes such as pancreatic lipase. Pancreatic lipase is a lipolytic enzyme that preferentially cleaves fatty acids at the *sn*-1 and *sn*-3 positions of TAG molecules. This is an important factor to consider when producing oils to be used as EFA sources. Generally, saturated fatty acids are preferentially placed at the *sn*-1 and *sn*-3 positions of TAG molecules while the *sn*-2 position tends to favour unsaturated fatty acids (including EFAs). Therefore, assuming that there is 100% placement of EFAs at the *sn*-2 position, an oil would have to contain in excess of 33.3% of the EFAs in order to ensure that they are efficiently utilized by the body. This would allow all of the *sn*-2 positions to be filled by EFAs and still have some remaining to be placed at the *sn*-1 and/or *sn*-3 positions where they can be effectively cleaved by pancreatic lipase and other such enzymes.

Essential Fatty Acids:

EFAs are those fatty acids that are required for optimal human health but are not synthesized by the body; they must be obtained from dietary sources. In humans

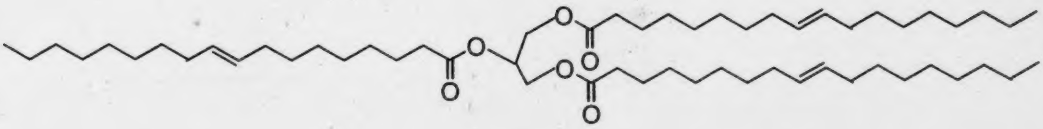

Figure 1-1: Triolein, an example of a TAG molecule. The three fatty acids that are attached to the glycerol backbone are all oleic acid. The topmost position in this diagram is designated the *sn*-1 position, the middle is the *sn*-2 position, and the bottom position is the *sn*-3.

polyunsaturated fatty acids (PUFAs) are not synthesized in sufficient amounts within the body, therefore, they must be obtained through external sources. For this reason they are often referred to as EFAs. There is some ambiguity in deciding which fatty acids are actually EFAs, since some of these molecules can be synthesized *in vivo* if their precursors are provided. However, some experts consider fatty acids that are directly linked to the eicosanoid pathways as EFAs (Figure 1-2). Figure 1-2a outlines the ω-3 pathway which is linked to the production of series 3 prostaglandins. Eicosapentaenoic acid (EPA) is a direct precursor to these eicosanoids and ALA (sometimes referred to as, simply, linolenic acid) is an ω-3 fatty acid that serves as a precursor to EPA. This ω-3 pathway then continues on to produce docosahexaenoic acid (DHA). DHA has been shown to be vital in the neural, retinal, and behavioral development of fish larvae and DHA deficiency in these larvae is known to be fatal. As in fish larvae, DHA is also known to be important in the neural development in human infants. Figure 1-2b outlines the ω-6 pathway which leads to the production of series 1 and series 2 prostaglandins. Dihommogamma-linolenic acid (DHGLA) is a direct precursor to series 1 prostaglandins while ARA is the immediate precursor to series 2 prostaglandins. *Gamma*-linolenic acid (GLA) is the immediate precursor to DHGLA and is also considered an EFA. LA is an ω-6 fatty acid that is a precursor to GLA, DHGLA, and ARA, as shown in Figure 1-2b. In this scheme, LA and ALA are not considered EFAs. However, according to the strict definition of an EFA, both of these fatty acids should not only be included in this grouping, they should be considered the only known EFAs since they both serve as precursors for the aforementioned molecules and, therefore, are ultimately eicosanoid precursors as well (3).

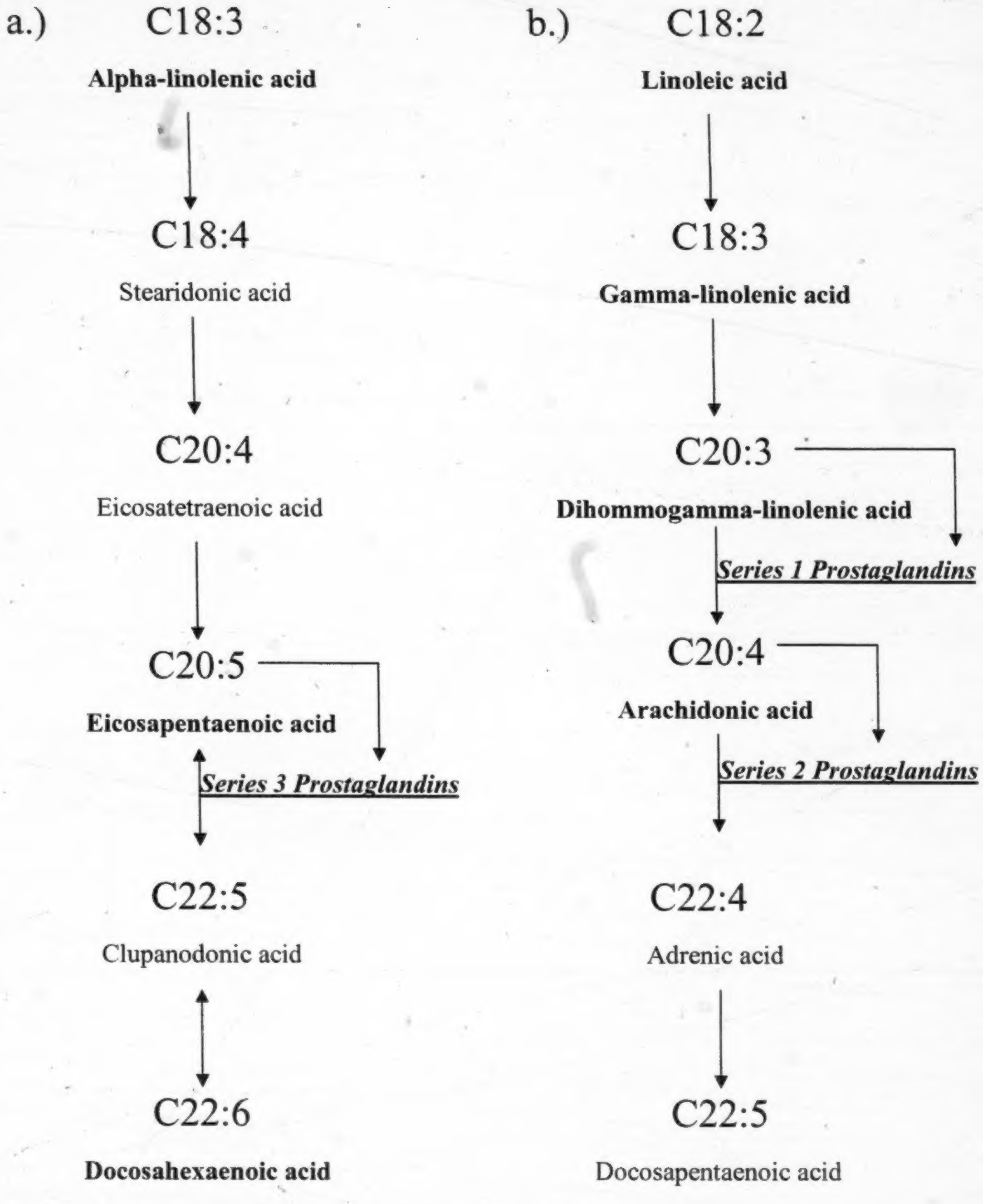
a.)
C18:3
Alpha-linolenic acid
C18:4
Stearidonic acid
C20:4
Eicosatetraenoic acid
C20:5
Eicosapentaenoic acid
Series 3 Prostaglandins
C22:5
Clupanodonic acid
C22:6
Docosahexaenoic acid
b.)
C18:2
Linoleic acid
C18:3
Gamma-linolenic acid
C20:3
Dihommogamma-linolenic acid
Series 1 Prostaglandins
C20:4
Arachidonic acid
Series 2 Prostaglandins
C22:4
Adrenic acid
C22:5
Docosapentaenoic acid

Figure 1-2: Overview of the EFA pathways. a) ω-3 pathway b) ω-6 pathway (EFAs in bold)

Eicosanoids (also collectively referred to as prostaglandins) are a class of biologically important molecules that have hormone-like actions in humans and include prostaglandins, thromboxanes, and leukotrienes (1). Prostaglandins are involved in smooth muscle constriction and include oxytocin, which is necessary for the contraction of the uterine wall during labour and for shedding of the endometrial lining during menstruation (3). Thromboxanes are also involved in smooth muscle constriction but they have the added effect of being involved in platelet aggregation (1). Leukotrienes play an important role in inflammation and allergic reactions (1). It is important to note that eicosanoids as a whole have various functions including ones that are antagonistic to each other (3). Also, though a class of eicosanoids may be derived from the same fatty acid precursor, they may not have analogous functions in the body. For example, the prostaglandin PGI2 helps to prevent platelet aggregation while PGE2 promotes platelet aggregation and both of these molecules are derived from ARA (3). Both of these compounds perform vital functions in the body that are important to the maintenance of homeostasis. Since eicosanoids derived from different EFAs can have similar functions, it is important that homeostasis be maintained. Therefore, an imbalance in one EFA can shift the homeostatic balance such that adverse effects can occur. For example, if too many eicosanoids that promote platelet aggregation are present without the presence of the eicosanoids responsible for the prevention of platelet aggregation to balance the effect, blood clots could form, causing circulatory problems.

The ω-3 fatty acids are generally perceived as being significant factors associated with the prevention and treatment of ailments related to the human circulatory system. (3, 4). For example, EPA, an ω-3 fatty acid, has been shown to prevent blood platelet aggregation and to reduce the risk of atherosclerosis. Since eicosanoids require EFAs as precursors, some symptoms of EFA deficiencies can be related to the lack of eicosanoid production. For example, LA deficiency has been found to cause a number of symptoms including failure to heal wounds, behavioral impairments, and heart and circulatory problems (3). The failure to heal wounds can be related to deficiencies in eicosanoids that aid in platelet aggregation. Heart and circulatory problems could result if eicosanoids that promote blood clotting are too abundant or if eicosanoids preventing platelet aggregation are too scarce. Although prolonged LA deficiency can be fatal, symptoms can be reversed by LA supplementation (3). ALA deficiency has been shown to cause behavioral changes, impaired vision, and impaired learning ability, among other symptoms (3). The aforementioned symptoms are consistent with those observed with DHA deficiency.

Sources of EFAs:

Since it is well known that EFA deficiencies can have negative effects on human health, some researchers have focused their attention on finding external sources of these fatty acids that can be produced inexpensively on an industrial scale. Currently, the main industrial sources of PUFAs are fish oils, although alternate sources are also used. Table 1-1 shows relative amounts of important EFAs in algal, yeast, and evening primrose oils (5, 6). ARA is also commercially isolated from animal livers while EPA is also extracted from algae and moss (5, 6). The major source of GLA, to date, is evening primrose oil (4, 5, 6). These current sources have relatively low PUFA yields and the production costs can be fairly high in relation to the amount of fatty acids that are extracted. As a result, some researchers have turned their attention to alternate PUFA sources from which fairly

Table 1-1: EFA profiles for some current sources (percentages are relative to the complete fatty acid profiles) (5, 6).

Fatty Acid	Algal sources (%)	Yeast (*S. cerevisiae*) (%)	Evening primrose oil (%)
GLA	0.20 – 2.60	0.07	3.00 – 15.00
DHGLA	0.50 – 5.20	0.00	0.00
ARA	0.04- 9.50	0.11	0.00
EPA	0.00 – 9.90	0.00	0.00

high yields of the target fatty acids can be extracted and which are relatively inexpensive. In an attempt to achieve this goal, recent research in this area has focused on oleaginous moulds as a possible alternative to current PUFA sources.

In recent years, there has been a marked increase in research interest involving the use of various moulds as potential producers of EFAs on an industrial scale. Some moulds have unique fatty acid compositions which contain PUFAs in relatively high amounts (7-10). Species belonging to the fungal genus *Mortierella* have attracted notable attention within this research area due to their potential as lipid producers. The EFAs that have received the most attention with regards to *Mortierella* research are ARA, EPA, GLA, and DHGLA (6, 11- 38). Research has also shown that the relative amounts of these EFAs can vary dramatically both between and within the *Mortierella* species. A number of factors, such as growth media composition, temperature, and pH, have been found to affect the fatty acid profiles of these fungi. More research must be done with regards to the optimization of these variables for industrial EFA production, however, sufficient research has been completed to warrant an overall review of this area.

Oleaginous Organisms:

Oleaginous fungi are defined as fungi that contain more than 25% of their biomass in the form of lipids (2). Research has shown that some moulds can store up to 80% of their biomass as lipids (2). As a general rule, oleaginous organisms have two distinct growth phases. The first phase consists of rapid cell proliferation that continues until one of the essential nutrients becomes a limiting growth factor. At this point of nutritional depletion, cell proliferation ceases. If carbon is available, the second stage of growth commences. During this stage anabolic lipid metabolism dominates, resulting in the accumulation of storage lipids. Once these lipids have accumulated, they can be mobilized when placed in a carbon-limited medium that is rich in nitrogen. This catabolism is triggered in order to allow for further mould growth (2). With regards to fungi of the *Mortierella* genus, studies have shown that temperature, pH, agitation, age of the mould and the composition of the growth media are all important factors in affecting mycelial growth and the fatty acid compositions of these moulds (6, 11-38).

Yeast Mutants as Cocoa Butter Substitute Producers:

Oleaginous moulds are also used to produce cocoa butter substitutes. Cocoa butter has a unique fatty acid composition and it is this unique profile that imparts the desirable traits of "snap" and "melt-in-mouth" to the chocolate into which it is incorporated. Unlike most vegetable oils (with the exception of lauric oils) cocoa butter has an exceptionally high saturated fatty acid content (approximately 60%). Approximately 35% of this is stearic acid (SA) while the remaining 25% is palmitic acid (PA).

There are two groups of cocoa butter replacers; cocoa butter equivalents (CBEs) and cocoa butter substitutes (CBSs). CBEs are oils that are designed to have compositions similar to cocoa butter. As a result, they have properties similar to cocoa butter and are designed to be completely compatible with cocoa butter in various chocolate mixtures. One example of a CBE can be obtained by interesterification of palm oil by using SA and a 1,3-specific-lipase. CBSs are oils that impart similar properties to the chocolate to which they are added but which have chemical compositions different from that of cocoa butter. As such, these oils can only be added to mixtures containing less than 20% cocoa butter. If the cocoa butter content exceeds this amount, the resulting chocolate has a tendency towards softening and/or bloom formation. Though the cost of production of these replacers are relatively low when compared to cocoa butter itself, industry producers are still looking for cheaper alternatives to both cocoa butter and its current replacements.

One possible alternative to current CBEs has been found in Δ9-desaturase (Ufa) yeast mutants, with a focus on the species *Cryptococcus curvatus* (formerly, *Apiotrichum curvatum*; originally, *Candida curvata* D) (39-44). The Δ9-desaturase enzyme is responsible for the conversion of SA to oleic acid (OA) and PA to palmitoleic acid (POA). Since the wildtypes of the yeast that are being studied as possible CBEs have fatty acid compositions that are higher in OA than cocoa butter, it is logical that the Ufa mutants are a more viable option. The lack of Δ9-desaturase activity in these mutants means that they have higher SA contents than their wild type counterparts and, therefore, have fatty acid compositions that are more like that of cocoa butter.

The substrate of choice for studies examining yeast-derived lipids as possible CBEs is whey permeate since it is a relatively inexpensive medium on which such yeast grow well. However initial studies with Ufa mutants found that the medium needed to be supplemented with OA in order for the appropriate fatty acid composition to be obtained. This supplementation of the medium adds another cost to the production of this oil.

Smit *et al.* (41) examined Ufa mutants of the yeast *Cryptococcus curvatus* to determine if there were any strains that could produce CBEs without the addition of OA to the culture medium. It was found that revertants of Ufa mutants could in fact be grown on unsupplemented media and still produce the required fatty acid profile. A previous study by Ykema *et al.* (44) also found that there were revertants that could produce a fatty acid composition comparable to cocoa butter, however, that study supplemented the media with OA. In an earlier study, Ykema *et al.* (42) found that whey permeate supplemented with rapeseed oil yielded growth and lipid production rates similar to the wild type while approaching a fatty acid composition similar to cocoa butter.

In addition to media supplementation, studies have been performed to determine the effect that the carbon to nitrogen (C/N) ratio has on lipid production in these fungi. Ykema *et al.* (43) found that the maximum lipid production was obtained at C/N ratios of 30-35 in whey permeate whereas the typical C/N ratio for this medium is between 70 and 101. This indicates that nitrogen supplementation may be necessary to optimize the lipid production in these fungi. However, this would also result in an additional cost to industrial producers.

Beavan *et al.* (39) screened 6,725 yeasts isolated from various sources and identified 1,757 as oleaginous. Further testing determined that a strain of *Trichosporon cutaneum* yielded the best results with regards to rates of carbon consumption, lipid production, lipid yield, and lipid composition. Fractionation of lipids obtained from the yeast yielded one fraction that showed a similar profile to that of cocoa butter and a second fraction that had a slightly higher melting point than that of cocoa butter. Both fractions were almost entirely composed of the most stable β crystal form. The effect that metal ions had on lipid production was also examined and it was found that limited metal ion supplementation resulted in lower lipid yields but higher lipid quality (i.e., with fatty acid profiles more similar to cocoa butter).

Although results look promising for this line of research, researchers have yet to make this a viable alternative on an industrial scale. In order for yeast mutants to be used on an industrial scale as CBSs, it is important that the production costs remain relatively low. This means that the media in which the yeast grow, as well as the equipment required for growth and oil extraction processes must be less expensive than those of current CBSs.

Algae as Sources of EFAs:

Algal sources of EFAs have primarily been used in the context of aquaculture (45), however, they have also captured interest as potential alternate EFA sources for human consumption. Miura *et al.* (46) studied the effect of various growth conditions on GLA production in a marine green alga which was found to produce high levels of GLA (*Chlorella* sp.). It was found that the optimum salinity for both GLA yield and biomass yield was 5 g/L while nitrogen starvation resulted in decreased GLA and overall unsaturated fatty acid yields.

Another green alga (*Parietochloris incisa*) was found to be a potent producer of ARA (47). At maximum lipid production, the ARA content of this alga made up to 50% of the total fatty acid yield which amounted to more than 20% of its biomass. It was also found that more than 90% of the ARA was deposited onto TAG molecules. This is an important fact to note since the distribution of the target EFA within TAG molecules would determine its bioavailability.

Fungi as Sources of EFAs:

Although plant sources are the main focus of research into alternate sources of EFAs for human consumption, fungal sources are also gaining popularity as potential sources. One of the main advantages of using fungi as an industrial source of EFAs instead of plant-derived products is that there would arguably be less competition for lands that would be needed to grow plant crops. If the optimum growth medium for the production of EFAs contains products that require little agricultural land, then the competition for land could be significantly reduced.

The second advantage of using fungi as EFA sources is that the fungi currently being studied have fatty acid profiles that are either naturally high in EFAs or can be induced to produce higher yields of the target fatty acids by abiotic manipulation of the growth medium. Such manipulation can be less invasive and time-consuming than performing genetic manipulations on the organism of interest. This also has the added benefit of avoiding potentially negative consumer attention in terms of GMO concerns.

The fungi that have garnered the most interest in the area of industrial EFA production are within the phylum Zygomycota, which is the second most basal group within the Kingdom Mycota. More specifically, the fungi of interest belong to the Order Mucorales. This group is best known for being saprophytic and probably the best-known member of this group is the *Rhizopus* genus (better known as common bread mould). The *Mucor* genus is another popular member of this order and some of its species are known to cause opportunistic infections in humans that are often referred to as either zygomycosis or, more specifically, mucormycosis. However, some *Mucor* spp. are also being studied as possible industrial EFA producers. One obvious question that arises is the safety of these fungi as potential food sources. In this matter, it seems that the *Mortierella* genus may have some advantages.

Although the most well-known member of the *Mortierella* genus is probably *M. wolfii* B.S. Mehrotra & Baijal, it is also the species that is least representative of the genus. This particular species is known to cause mycotic abortions in cattle and, although no human infections have been recorded, there is also some speculation as to the possible effects it could have on humans (48-50). Unlike this rogue member, all other *Mortierella* spp. are known to be benign and do not cause even opportunistic infections in humans. Furthermore, studies have been done testing the safety of oil produced by *M. alpina* Peyronel which was then fed to rats (36, 51, 52). The results were definitive; the fungal oil is completely safe for human consumption. There is little reason to believe that the results for toxicity testing would be any different for oils obtained from other *Mortierella* spp. being studied for possible industrial scale EFA production. However, the testing must still be done.

The genus *Mortierella* can be further subdivided into two subgenera based mainly on their individual and colonial morphologies (53). The two subgenera are *Mortierella* and *Micromucor*. Since one of the main differences between these two subgenera is in the fatty acid biosynthetic pathways, it is important to note which species fall into which subgenus in order to determine which fatty acids can be potentially targeted for industrial production in a given species. Amano *et al.* (7) examined the fatty acid compositions of various moulds within the genus *Mortierella*. Prior to the Amano *et al.* (7) study, the various species within the genus *Mortierella* were placed into either of these subgenera on the basis of the morphological and cultural attributes that they exhibited. However, this method of subgenera identification was not comprehensively definitive. The main objective of the Amano *et al.* (7) study was to determine whether there were differences in the fatty acids that were found within this genus that would allow for comprehensive identification of moulds belonging to either subgenera. Results showed that there were, in fact, differences in fatty acid composition between these two taxonomic groups that would allow for definitive classification of these moulds into their respective subgenera. It was found that fungi belonging to the subgenus *Mortierella* contained C20 PUFAs while those in the subgenus *Micromucor* did not. Some species that are found within the subgenus *Micromucor* include *Mortierella ramanniana* (A. Moller) Linnem, *M. isabellina* Oudem, and *M. vinacea* Dixon-Stew. Some fungi that belong to the subgenus *Mortierella* include *M. alpina*, *M. elongate* Linnem, and *M. hyalina* (Harz) W. Gams. The results from the above studies imply that the best sources for C20 EFAs (e.g., ARA, EPA, etc.) within the genus *Mortierella* would be found in the subgenus *Mortierella* while the subgenus *Micromucor* would be the best source for the C18 EFAs (e.g., ALA, GLA, etc.).

The fatty acid compositions of fungi from the genus *Mortierella* can be manipulated by varying the conditions under which these fungi are grown. However, before the compositions are manipulated, the original distribution of fatty acids within the mould of interest must be known to provide a reference point. In the study by Amano *et al.* (7), the fatty acid compositions of eighteen moulds from the subgenus *Micromucor* and fifty moulds from the subgenus *Mortierella* were determined. The moulds used for fatty acid analyses were cultured in a glucose (2%) and yeast extract (1%) liquid medium with a pH of 6.0 and were grown at 28 °C for between four to seven days. Most notably, with respect to PUFA research, the eleven *M. alpina* strains tested contained between 20.9% and 69.7% ARA and between 3.1% and 6.5% DHGLA. Table 1-2 shows the relative amounts of fatty acids found in three species of the genus *Mortierella*. The ranges outlined in Table 1-2 represent the variations reported for various strains within each species. The two *M. hyalina* strains that were used in this study had lower

Table 1-2: Fatty acid compositions obtained by Amano *et al.* (7) for three fungi from the *Mortierella* subgenus. The ranges shown for each species represents the variations observed for a number of different strains within that species.

Fatty Acid	*M. alpina* (%)	*M. hyalina* (%)	*M. elongata* (%)
Palmitic	6.6 - 20.3	17.3 – 22.5	15.4 – 19.0
Stearic	4.0 – 9.9	6.7 – 12.9	8.3 – 14.0
Oleic	6.9 – 30.5	29.3 – 36.5	30.3 – 33.3
LA	4.9 – 14.4	7.4 – 9.6	6.7 – 7.4
GLA	3.8 – 10.9	7.3 – 7.5	6.0 – 7.4
DHGLA	3.1 – 6.5	3.1 – 3.3	3.6 – 4.9
ARA	20.9 – 69.7	11.3- 14.4	14.3 – 22.8

Table 1-3: Fatty acid compositions obtained by Amano *et al.* (7) for three fungi from the *Micromucor* subgenus. The ranges shown for each species represents the variations observed for a number of different strains within that species.

Fatty Acid	*M. ramanniana* (%)	*M. isabellina* (%)	*M. vinacea* (%)
Palmitic	16.0 – 25.2	15.6 – 21.4	18.1
Stearic	1.7 – 5.4	1.7 – 4.3	2.2
Oleic	21.4 – 41.7	38.3 – 51.9	46.3
LA	16.1 – 25.0	11.6 – 17.9	11.4
GLA	13.2 – 31.4	6.9 – 22.9	22.0

percentages of ARA and DHGLA while the amount of OA was higher overall when compared to that which was found in the *M. alpina* strains. The percentages of the other fatty acids fell within the same range as those reported for the *M. alpina* strains (refer to Table 1-2). A third *Mortierella* species, *M. elongata*, had a fatty acid composition that consisted of relative percentages of fatty acids that fell within the range of both of the previously mentioned species, with the exception of SA which was slightly higher for one of the strains tested. These results are also outlined in Table 1-2.

In contrast to the isolates from the subgenus *Mortierella*, fungi from the subgenus *Micromucor* tended to have higher OA contents while the other fatty acids had considerable variability between species, as well as between strains as shown in Table 1-3 (7). The species *M. isabellina*, also showed variation between strains, however, there was a generally higher OA content (38.3% - 51.9%) when compared to *M. ramanniana* strains (21.124% - 41.7%). As before, the ranges reported in Table 1-3 represent variations between the strains of a given species studied.

In a study by Eroshin *et al.* (12), various *Mortierella* species were tested for ARA-producing capabilities using an aspirin-containing medium. The control cultures were grown on a solid potato-dextrose-agar (PDA) medium without aspirin for fifteen days. Figure 1-3 shows the average lipid yield of each species with variations between strains represented by the standard deviation expressed as error bars. With regards to percentage of lipid per dry biomass, the various strains of *M. isabellina* yielded higher lipid content than the *M. alpina* strains. In fact, one of the *M. isabellina* strains actually had the highest percentage of lipid yield overall. The single *M. vinacea* strain which was used in this study also contained a relatively high amount of lipid while the *M. elongata*

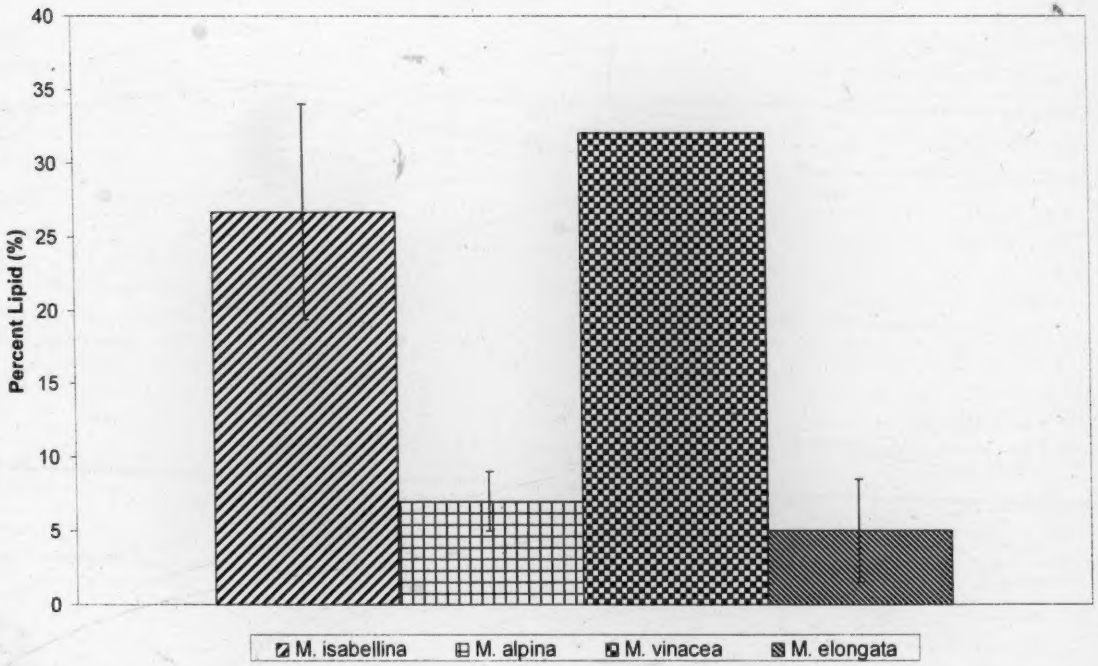

Figure 1-3: Average percent lipid yield for various *Mortierella* spp.(12). Error bars represent variations in observed lipid percentages between different strains of the same species. Only a single *M. vinacea* strain was studied.

strains had lipid contents similar to those found in the *M. alpina* strains. Figure 1-4 shows the average percentage of specific fatty acids found within various species with the fatty acid variations between strains of the same species being represented by the standard deviation to the mean and expressed as error bars. Figure 1-4a represents a study by Amano *et al.* (7) and 1-4b, the study by Eroshin *et al.* (12). With regards to the fatty acid compositions, the amounts and ranges were similar to those found by Amano *et al.* (1992) for *M. alpina* with the exception of OA which had a broader range than was found in the Eroshin *et al.* (12) study. LA in the Eroshin *et al.* (12) study also had a slightly larger range than was reported by Amano *et al.* (7). The fatty acid compositions of *M. isabellina*, *M. vinacea*, and *M. elongata* all had substantial differences when compared to the results obtained from the Amano study. First, there were higher values reported by Eroshin *et al.* (12) in the OA and LA content in *M. isabellina*. Secondly, there were dramatic differences in the fatty acid composition of *M. vinacea* between the Amano and Eroshin studies. Namely, Eroshin *et al.* (12) reported higher percentage values for palmitic, stearic, and linoleic acids and lower values for the relative percentages of oleic and linolenic acids. Furthermore, the reported values by Amano *et al.* (7) imply that only the C16 and C18 fatty acids are present in *M. vinacea* whereas Eroshin *et al.* (12) report that C14:0 and C15:0 are also present in this mould species. An interesting comparison is found within the species *M. elongata*. While it can be observed that there are slightly higher percentage values for PA and OA in the Eroshin *et al.* (12) paper, a particularly intriguing observation can be made when comparing the reported values for *M. elongata* within the Eroshin *et al.* (12) study itself. There is one strain of this mould that is markedly different from its counterparts in that it has an extremely high

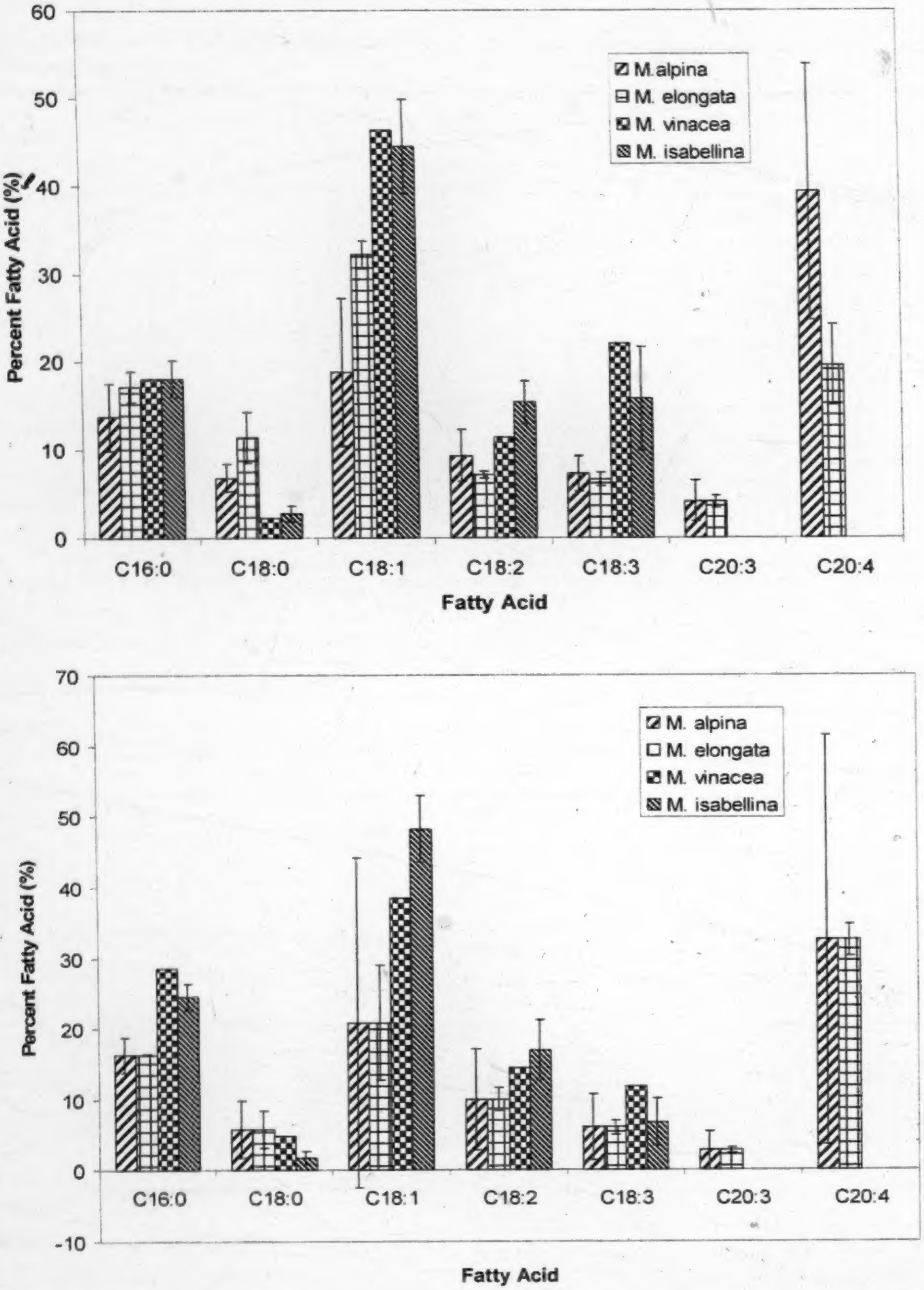

Figure 1-4: Comparisons of average fatty acid yields as percentages for various *Mortierella* species between two studies. Amano *et al.* (7) b) Eroshin *et al.* (12). Error bars represent variations in reported fatty acid percentages between different strains of the same species. Only a single *M. vinacea* strain was studied in each case.

OA content (45.7%) and a significantly lower LA content (0.6%) when compared to other strains of the same mould species.

In comparing the results from these two studies, it is important to note the differences between the methodologies (i.e., growth conditions and analytical procedures) themselves. First, each study reported slightly different components of the fatty acid compositions. More specifically, the study by Amano *et al.* (7) did not report the relative percentages of C14:0, C15:0, or C20:1 as separate entities as did Eroshin *et al.* (7) but rather reported them under the collective heading of "others". Also, C16:1 was not reported at all in the Amano *et al.* (7) study. Conversely, the "others" category in the Amano *et al.* (7) study included fatty acids that were not reported in the paper by Eroshin *et al.* (12). Secondly, there were differences in the media used for each study. Thirdly, each set of researchers used different strains of fungi from the same species. These variations in methodology and reporting can account for some of the variations found between the fatty acid compositions reported by these two sets of researchers. More importantly, the differences reported in the fatty acid profiles of these two groups suggest that there are a number of controllable factors which may significantly alter the fatty acid profiles of these mould species.

Other researchers have also reported on the fatty acid composition of various *Mortierella* mould species and, as noted above, differences in methodology and reporting must be taken into account when comparing the data from different studies. In a study by Jansa *et al.* (9), various fungi, including some from the *Mortierella* genus, were grown in a malt extract liquid medium and their resulting fatty acid compositions were determined. The composition of *M. alpina* was within the ranges reported by Eroshin *et al.* (12) and

Amano *et al.* (7) with the exception of fatty acids with chain lengths exceeding twenty carbons. Neither of the previous studies reported any fatty acids having chains longer than twenty carbons in length. However, Jansa *et al.* (9) reported that these longer chain fatty acids comprise 3.7% of the fatty acid composition of *M. alpina*. The fatty acid composition of *M. elongata* was similar to those previously reported with the inclusion that longer chain fatty acids accounted for 1.7% of the total fatty acids reported. Jansa *et al.* (9) also reported on the fatty acid composition of a mould that they refer to as *Micromucor ramannianus* var. *ramannianus*. Although it is not explicitly stated, the assumption is that this refers to the previously named *Mortierella ramanniana* var. *ramanniana* which has been cited in the article by Amano *et al.* (7). Based on this assumption, a comparison can be made between the relative percentage values reported for the fatty acid compositions of these mould species. Generally, the values for the various fatty acid species agree with the exception of a slightly higher reported percentage for OA by Jansa *et al.* (9) which is seemingly compensated by a slightly lower reported value for the remaining C18 fatty acids. Also, the fatty acid composition includes a percentage of longer chain fatty acids (1.4%) which had not been mentioned in the previously cited study.

Myher *et al.* (54) also conducted a study in which a more complete fatty acid composition was reported for polyunsaturated oils obtained from various sources, including three strains of *M. alpina*. The growth conditions of the mould species used in this study were not included in the paper, however, once the differences in reporting are accounted for, it can be noted that the reported percentages fell within the values outlined for the previous three studies.

Effect of Growth Conditions:

Effect of pH, Temperature, and Aging:

Once the basic fatty acid compositions of various moulds of interest have been outlined, as briefly summarized above, the next important step is to examine what effect various growth conditions have on the fatty acid composition as well as gross lipid yield per unit biomass produced. Several studies have been performed in this area using several species of fungi from the genus *Mortierella* as the organisms of interest. This is especially vital when attempting to optimize growth conditions for a given EFA within a given *Mortierella* strain. These growth factors include pH, temperature, aging, carbon source, nitrogen source, metal ion types, and supplementation of the growth medium.

Hansson and Dostalek (14) studied the effect that incubation temperature had on biomass yield and fatty acid composition. They examined the growth of two *Mortierella ramanniana* strains, *M. vinacea* , and *M. isabellina* Oudem. at 20 °C, 25 °C, and 30 °C. It was observed that 25 °C was the optimal growth temperature for maximum biomass yield. There was some variation noted in fatty acid composition with respect to incubation time and temperature. Generally, the degree of unsaturation increased as temperature decreased. This unsaturation was affected by the fact that the GLA content of the moulds increased with a decrease in temperature.

Lower growth temperatures tend to favour the production of unsaturated fatty acids. Results from a study by Shimizu *et al.* (30) also support this observation. In this study, the effect that temperature had on the fatty acid composition of *Mortierella* fungi which normally produce ARA but not EPA at 28 °C was examined. The species studied included *M. hygrophila* Linnem, *M. zychae* Linnem, *M. elongata, M. parvispora* Linnem, *M. schmuckeri* Linnem, and *M. alpina* and selected results are shown in Figure 1-5. It was observed that the optimum temperature for ARA production in *M. alpina* was 28 °C while it was found that EPA production was stimulated by lower temperatures. Although EPA accumulation occurred up to 20°C, there was a significant decrease in EPA content when the growth temperature was above 12°C. Generally, ARA content decreased with decreasing temperature whereas EPA content increased with decreasing temperature. This suggests that ARA and EPA production are related by a metabolic pathway. Shimizu *et al*. (30) suggest the presence of an enzyme catalyzed reaction that is activated at low temperatures which converts ARA to EPA.

Nakahara *et al*. (26) investigated the temperature effect on the fatty acid composition of *Mortierella* fungi in more detail by determining the fatty acid composition of both the polar and neutral lipid fractions. Within the species *M. isabellina* this study found that regardless of temperature, the polar lipid fractions contained more unsaturated fatty acids overall than the neutral lipid fractions. Furthermore, contrary to previous studies on other species of *Mortierella* fungi, GLA content actually decreased in both the neutral and polar lipid fractions with decreasing temperature. Meanwhile, results from the fatty acid compositions of *M. ramanniana* var. *angulispora* grown at various temperatures indicate that the GLA content of the polar lipid fractions tended to increase with decreasing temperature while the GLA content of the neutral lipid components remained relatively consistent regardless of temperature.

Leman and Brakoniecka-Sikorska (24) studied the effects that cultivation time, pH, and temperature had on the IFO 8187 strain of *M. ramanniana* var. *angulispora*. With regards to temperature (at pH 4.5), the saturated and monounsaturated fatty acids

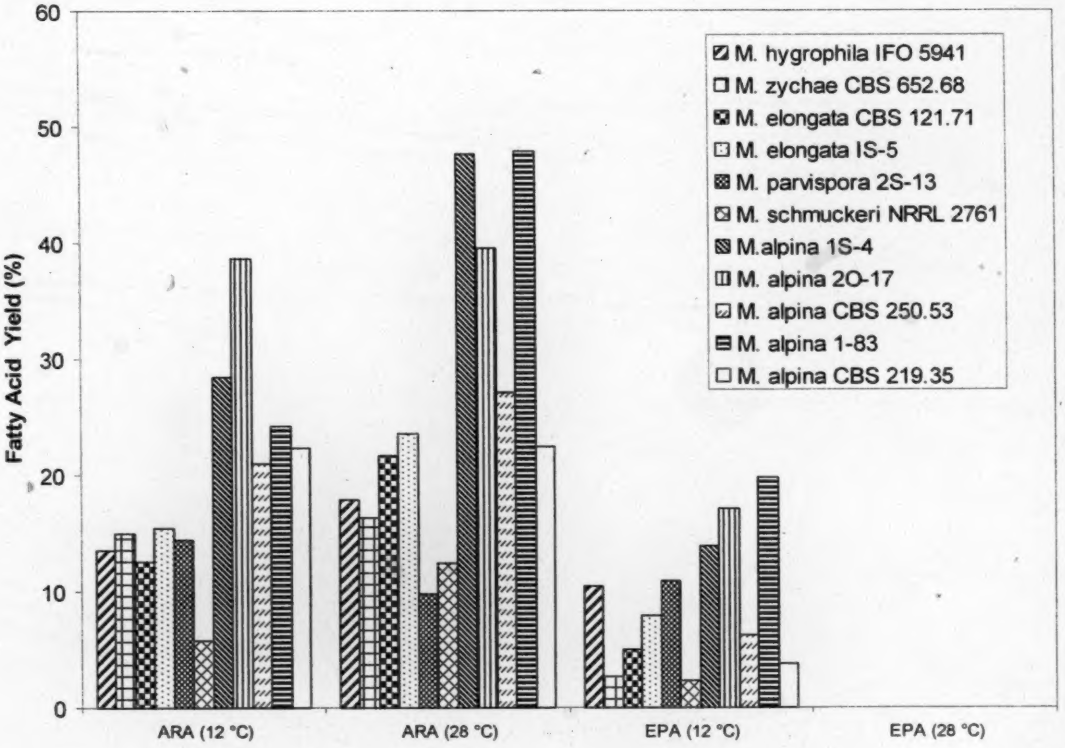

Figure 1-5: ARA and EPA yields for various *Mortierella* species grown at different temperatures (30). There was no EPA produced at 28 °C for any of the strains studied

reported tended to be present in greater amounts at 35 °C than at 25 °C. Conversely, the reported PUFAs had a tendency to be present in lower amounts at 35 °C than at 25 °C. This result is consistent with the previously stated observation that lower growth temperatures favour the production of PUFAs within *Mortierella* fungi. When comparing the fatty acid composition of *M. ramanniana* var. *angulispora* (Naumov) Linnem with respect to varying pH values, it was generally found that there was a tendency for the amounts of saturated and monounsaturated fatty acids to decrease with increasing pH values. The PUFA content, on the other hand, tended to increase with increasing pH values. The final factor, incubation time, appeared to have inconsistent effects on the fatty acid compositions and no obvious trends were observed.

The Leman (24) study found that, with regards to temperature, there did not appear to be any significant differences in the fatty acid composition over time. There were fluctuations found with cultivation time with respect to varying pH values, however, these differences were erratic and, therefore, do not point to any specific pattern of fatty acid accumulation over time. Other studies have specifically examined the effect that aging has on the fatty acid compositions of various fungi. One such study was performed by Shimizu *et al.* (31) on several ARA-producing fungi from the *Mortierella* genus. The effect of aging was studied in three strains of *M. alpina* under two sets of conditions. In the first condition, the harvested mycelia were divided into two groups. One set of mycelia were analyzed for their fatty acid compositions while the second set was allowed to stand at 28 °C for an additional seven days. Secondly, the harvested mycelia were once again divided into two groups. As in the previous step, one set of mycelia underwent fatty acid analysis, however, the second group was cultivated for an additional seven days at 28 °C. There were no significant differences between the mycelial mass before or after aging in any of the isolates. There were, however, comparatively large changes in the fatty acid compositions in each group of isolates and within each condition. Regardless of the condition, each strain showed marked increases in their EPA and ARA contents after aging. Upon closer inspection, increases in both EPA and ARA content after aging are greater in the moulds subjected to the first set of conditions than those that underwent the second set of conditions. It is interesting to note that the yields of both of these PUFAs are greater when the fungi are allowed to merely stand rather than being cultivated for an additional few days. Presumably, this is due to the fact that the fungi stop accumulating biomass and produce lipids since they no longer have access to a growth medium containing the building blocks for growth.

In another study, the effect of aging on the distribution of ARA within various lipid classes (i.e., phospholipids, free fatty acids, TAGs, and steryl esters) was examined using five fungal species (*M. hyalina, Cunninghamella elegans* Lendn, *Thamnidium elegans* Link, *Syncephalastrum racemosus* Cohn exJ. Schrot, and *Absidia anomala* Hesselt & J.J. Ellis) (55). The mould cultures were grown in potato-dextrose-broth (PDB) and samples were harvested at various times during cultivation for fatty acid analyses. Figure 1-6 shows that the total lipid contents of each fungal species increased with age. The neutral lipid fraction was responsible for this increase (55). This conclusion was reached based on the observation that there were observable increases in the relative proportions of the TAG and steryl ester components of the neutral lipid fraction whereas there were no significant changes occurred within the polar lipid components. With regards to the fatty acid distribution between the various lipid

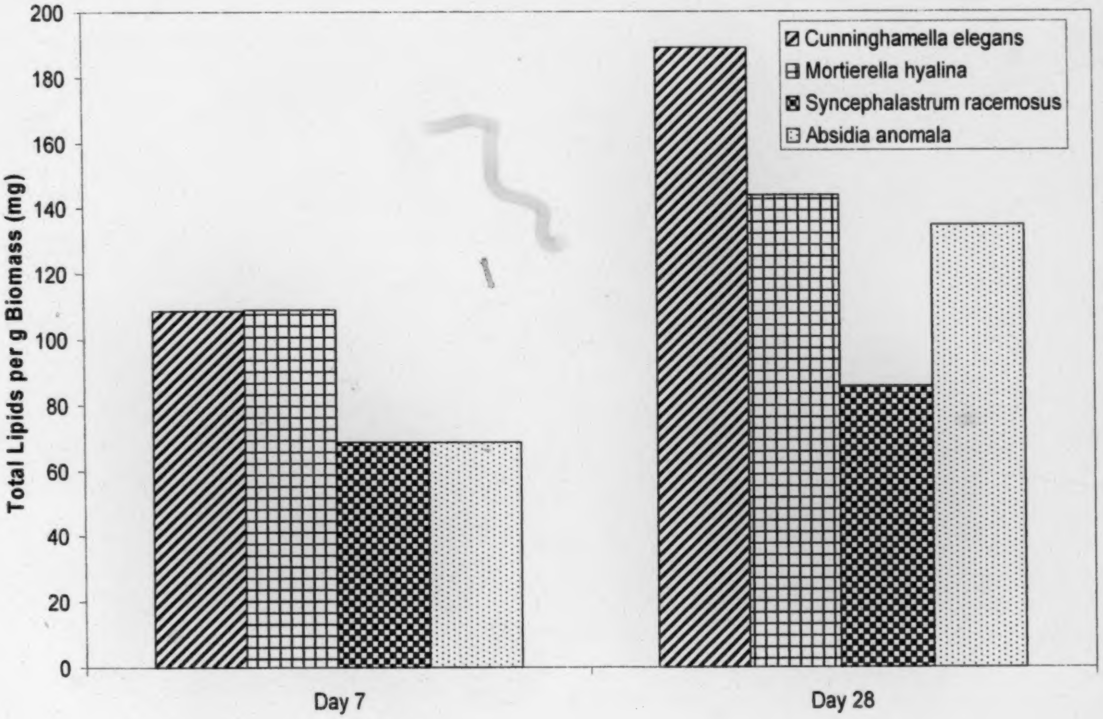

Figure 1-6: Lipid yield per g biomass for various fungal species over 28 days growth (55).

components, it was found that for *M. hyalina*, the phospholipid fraction contained the highest ARA content, regardless of age. It is also interesting to note that, while the ARA content within the phospholipid fraction decreased with age, the ARA content within the free fatty acid and TAG components increased with age. Similarly, while the GLA content decreased in the phospholipid fraction during aging, it increased with age within the TAG component.

Kendrick and Ratledge (20) performed a study in which they also examined the lipid content of various lipid components within a number of different fungal species, including *M. alpina-peyron*. Unlike Radwan *et al.* (55), Kendrick and Ratledge (20) reported quantitative values for the lipid content of each lipid fraction. Within *M. alpina-peyron*, 52.6% of the lipid content was found in the neutral fraction, 28.9% was found in the phospholipid fraction, and the remaining 18.4% of the lipids were found in the sphingolipids plus glycolipids fraction. None of the *M. alpina-peyron* fractions contained any EPA, however, each fraction did contain both GLA and ARA in varying amounts. The highest ARA content was found in the phospholipid fraction which was approximately twice that found in the neutral lipid fraction and almost ten times that found in the sphingolipid-glycolipid component. The phospholipid fraction also contained the highest GLA content when compared to the other lipid components. However, the neutral lipid fraction contained almost twice as much DHGLA when compared to the phospholipid component and this fatty acid was not present at all in the sphingolipid-glycolipid fraction.

Generally, the trends observed with temperature as a variable showed that lower temperatures favoured the production of unsaturated fatty acids when compared to higher temperatures. In the context of EFAs, this means that one would expect lower temperatures to favour EFA production. However, the biomass and lipid yields obtained at lower temperatures need to be taken into account when deciding what temperature is optimum for EFA production. If the biomass and/or lipid yields are too low at lower temperatures, the conditions may not be ultimately ideal for EFA production although EFA accumulation per unit biomass may be at a maximum.

The results from the aging experiments seem to indicate that harvesting fungal mycelia and allowing them to sit for a few days actually increases lipid accumulation when compared to cultures that are allowed to grow for the entire period of time. However, in some cases, the EFA of interest also decreases in yield over this period of time. Therefore, depending on the fungal strain and EFA of interest, aging of the fungi in this manner may or may not be an optimum condition for EFA production. Similarly, the results from pH experiments appear to vary with fungal strain.

Effect of Varying Carbon, Nitrogen, and Metal Ion Source:

A study performed by Sajbidor *et al.* (10) examined the effect of various liquid media on the fatty acid composition of four fungal strains, including *M. ramanniana*. There were six different media, each containing a different carbon source (maltose, lactose, glucose, soluble starch, glycerol, or sodium acetate). The pH of each medium was adjusted to 6.1 and the moulds were allowed to grow for seven days at 25 °C on a rotary shaker. The results for *M. ramanniana* showed that glucose gave the best mycelial yield (46.1 g/100 g substrate) while sodium acetate gave the poorest mycelial yield (0.2 g/100 g substrate). The remaining four substrates gave yields between 22.3 g/100 g substrate and 37.2 g/100 g substrate. However, with regards to the total lipid in dry biomass, sodium acetate gave the highest percentage (17.4%) while maltose had the lowest lipid yield (7.1%). Glucose was intermediate with a lipid yield of 14.0%. When taking into account both the mycelial and lipid yields on any given medium, glucose still gives the best results when compared to the other carbon sources. When comparing the fatty acid compositions of *M. ramanniana* grown on each of these media, it was found that sodium acetate yielded the highest percentages of palmitic, palmitoleic, stearic, and oleic acids whereas it resulted in the lowest yields of LA and GLA. Figure 1-7 shows the GLA yield per g biomass. The highest percentage of LA was found in the moulds grown in the lactose-containing medium while the highest GLA content was found using the soluble starch medium. Glucose yielded intermediary results for all fatty acids reported.

As the above study shows, there are several variables that must be examined before deciding which conditions best suit the required fatty acid needs. In the case of the study outlined above, the researchers were specifically looking at the production of GLA within each of their moulds. For *M. ramanniana*, the best option may be to grow the mould in a liquid medium containing soluble starch as the carbon source since this type of growth medium yields the highest GLA percentage with a relatively minor loss in mycelial yield when compared to the glucose medium. On the other hand, when compared to the soluble starch medium, glucose results in a slightly higher mycelial yield with a relatively low GLA yield overall.

In a similar type of study by Buranova *et al.* (8), the fatty acid compositions of various fungal strains from the *Mortierella* genus grown on two different media were determined. Once again, the PUFA of interest was GLA. The two media used varied in more than just their carbon sources (metal ion type and concentrations), however, the pH

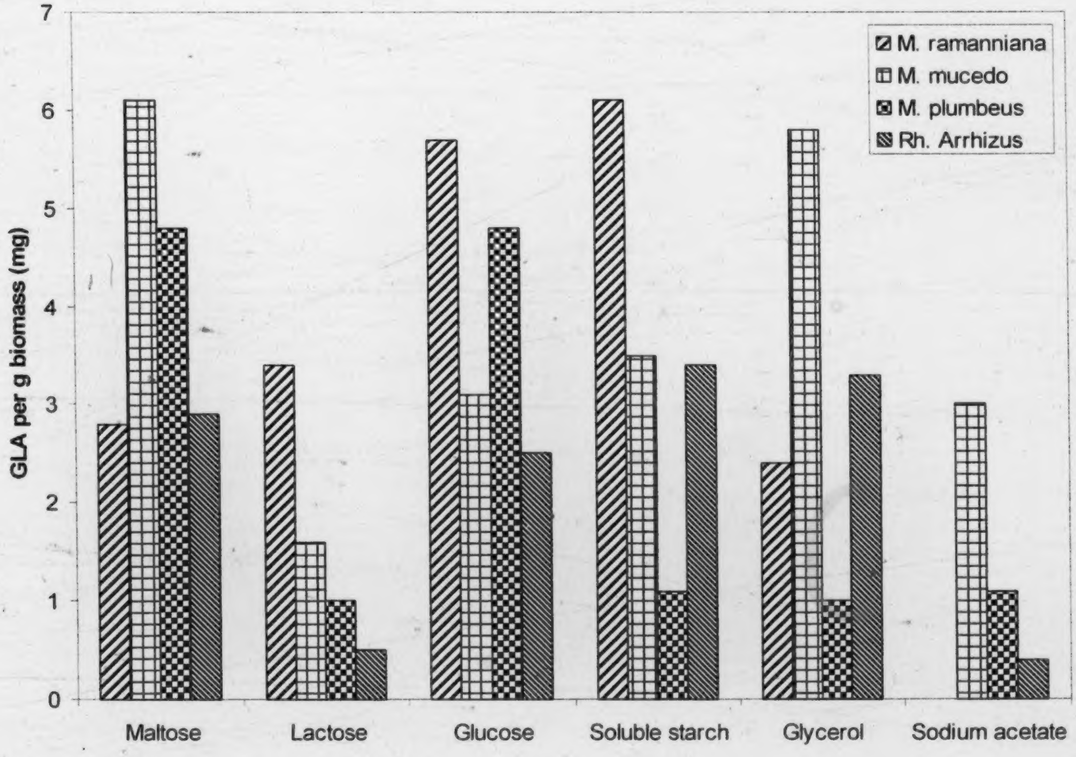

Figure 1-7: GLA per g biomass for various Zygomycetes species utilizing various carbon sources (10)

of each medium was adjusted to 5.7 and the moulds were all cultivated at 28 °C for seven days in a reciprocal shaker. The two carbon sources used were glucose and malt extract. There was a marked increase in biomass for all strains when the moulds were grown in the malt extract-containing medium. With regards to fatty acid yield (g/L substrate), there were lower values reported for all strains of *M. ramanniana* when they were grown in the malt extract containing media while there were no significant differences in the yields obtained by *M. vinacea*. The four strains of *M. isabellina*, however, varied in their results. Two strains of *M. isabellina* had slight decreases in their fatty acid contents while the remaining two strains had marked increases in these reported yields.

With regards to the fatty acid compositions, the *M. ramanniana* strains showed an overall decrease in the percentages of SA and a slight overall decrease in PA when they were grown in the malt extract medium when compared to the glucose medium. However, it is interesting to note that although there was an overall marked increase in the percentage of GLA in the reported fatty acid composition for the moulds grown in the malt extract media, Figure 1-8 shows that the GLA content (mg/L substrate) actually decreased for all but strain 224. This is in contrast to the increase in GLA content found in the *M. isabellina* and *M. vinacea* strains. These results support the observation that was noted previously indicating that both biomass (or mycelial) yield and fatty acid composition are important factors in determining which mould gives the best yield for a particular target molecule. Buranova *et al.* (8) showed that, the appropriate carbon source, with regards to GLA yield, varies with the mould strain.

A study by Hansson and Dostalek (14) examined the effect that carbon and nitrogen sources had on the production of GLA by *M. ramanniana*, *M. vinacea*, and *M.*

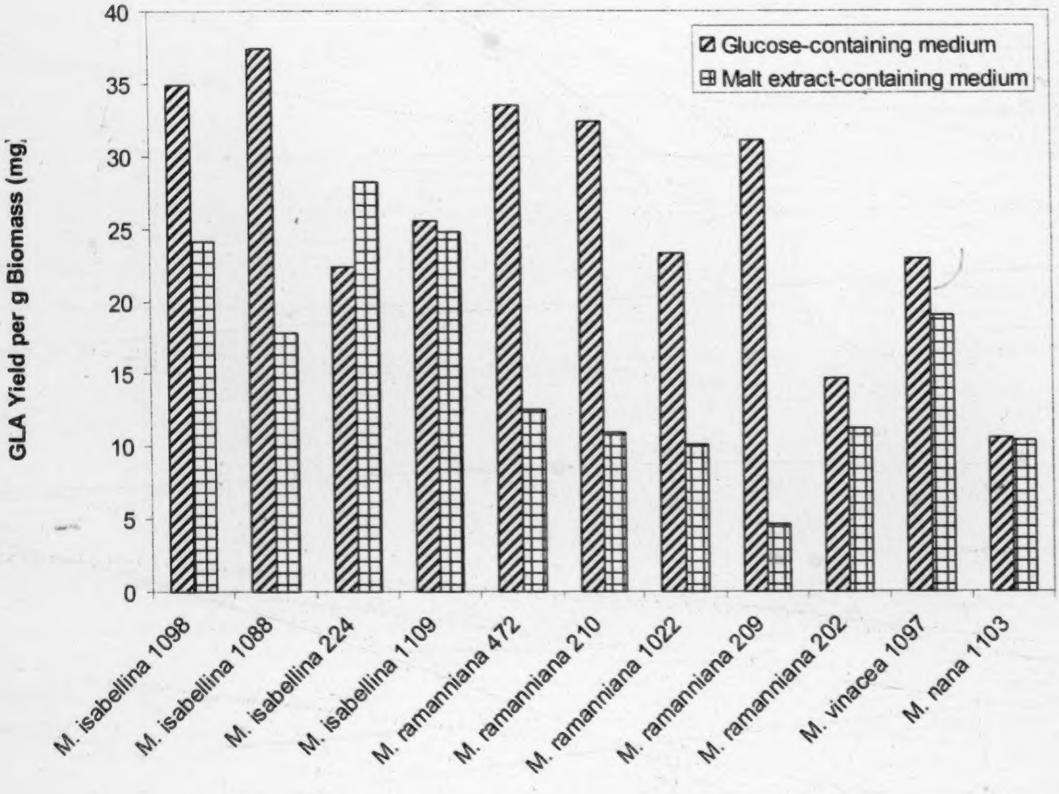

Figure 1-8: GLA per g biomass for various *Mortierella* species utilizing a glucose-containing medium and a malt extract-containing medium (8)

isabellina. Generally, it as found that carbon source had little effect on the fatty acid compositions of these fungi. With regards to nitrogen sources, it was observed that inorganic sources (e.g., KNO_3) were better suited for lipid accumulation than organic sources (e.g., amino acids). Furthermore, results showed that the addition of Cu-ions and Zn-ions had a stimulatory effect on both lipid and GLA production whereas the addition of manganese did not. Further increases in the amount of magnesium over and above what was already in the growth medium also had no marked effect on either of these components.

A study by Sajbidor *et al.* (27) investigated the effects of metal ions on lipid yield and ARA production in *Mortierella* sp. S-17. The basal medium consisted of Czapek's liquid medium with 5 g/L yeast extract and 20 g/L glucose. Results from this study indicated that both lipid and ARA yields were maximized with the addition of 2 mg/L Mn^{2+} to the basal medium while lipid production was minimized with the addition of 800 mg/L Fe^{2+}.

In order to explore the effect of growth media composition, Shinmen *et al.* (33) systematically studied the effects of carbon source and nitrogen source on the mycelial growth and ARA content of the 1S-4 strain of *M. alpina*. This particular strain had been chosen for an indepth study based on its high ARA production during a screening study. It was found that this strain grew rapidly in glucose containing media when either yeast extract or corn steep liquor was used as a nitrogen source. Furthermore, fructose, maltose, soluble starch, and corn starch all gave similar mycelial yields and ARA contents as those obtained with glucose. The optimum glucose concentration for ARA production was found to be 2-4% with yeast extract as the nitrogen source and 2% with corn steep liquor as the nitrogen source. When *n*-octadecane or *n*-hexadecane were used, the ARA yields were 1.2 – 1.5 times higher than those obtained with glucose, however, mycelial growth was poor. In regards to nitrogen source, it was found that yeast extract, corn steep liquor, and soybean meal were appropriate nitrogen sources for ARA production. The optimum concentration of yeast extract when glucose was used as a carbon source was 1%. It is important to note that although higher mycelial yields were obtained with higher concentrations of yeast extract, the ARA contents significantly decreased.

Stredanksa and Sajbidor (35) performed a similar study using *M. alpina* CCF 185 as the test organism. The results indicated that glucose was the best carbon source for lipid production though dextrin and starch (which are both glucose polymers) were also efficiently converted to lipid. The best nitrogen source for both lipid and ARA production was found to be yeast extract. These results support those reported by Shinmen *et al.* (33).

Leman (23) studied the effects that glucose concentration had on GLA production by *M. ramanniana* var. *angulispora* IFO 8187. It was found that GLA production increased with increasing glucose concentration however the overall GLA yield was maximized at the lowest glucose concentration (5%) due to the higher lipid yield. Results also indicated that GLA production was minimized in nitrogen-limited medium.

These studies show that carbon and nitrogen sources are utilized differently by different fungal strains. The optimum carbon and/or nitrogen source and concentration for one fungal strain cannot be generalized to other strains within its genus or even its species. Even so, it appears that glucose and yeast extract were always efficiently utilized by all fungal strains studied. However, their performance in relation to other carbon and nitrogen sources tested varied across strains and conditions.

Effect of Supplementation of the Growth Medium:

In addition to varying the composition of the growth medium, research has also been done which involves supplementing media with various compounds that could serve as precursors for certain target molecules. An example of this type of study, performed by Bajpai *et al.* (11), focused on the effect of the manipulation of growth conditions on EPA production by various *M. alpina* and *M. elongata* strains. The initial stage of the experiment involved screening the various *Mortierella* strains using three types of glucose-containing media containing 1% linseed oil. The purpose of this portion of the study was to determine which media gave the highest EPA yields, thereby allowing the researchers to select the appropriate medium to be used in the subsequent phases of this study. The next stage of the experiment involved cultivating the various fungal strains on media that either contained or did not contain linseed oil. Figure 1-9 shows the percent EPA produced with and without linseed oil supplementation of the growth media. Within the *M. elongata* species, the addition of linseed oil caused an increase in the percentage of both EPA and LA. Conversely, there were marked decreases in the percentages of palmitic, stearic, oleic, and arachidonic acids in the moulds grown in the linseed oil containing media. With regards to the *M. alpina* strains, similar results were obtained with one notable acception; EPA production only occurred in the presence of linseed oil in the growth media for two out of the three strains of *M. alpina*. Furthermore, in the third *M. alpina* strain, neither ARA nor EPA was produced when the moulds were grown in either type of media. Another interesting point to note is that incubation at 11 °C

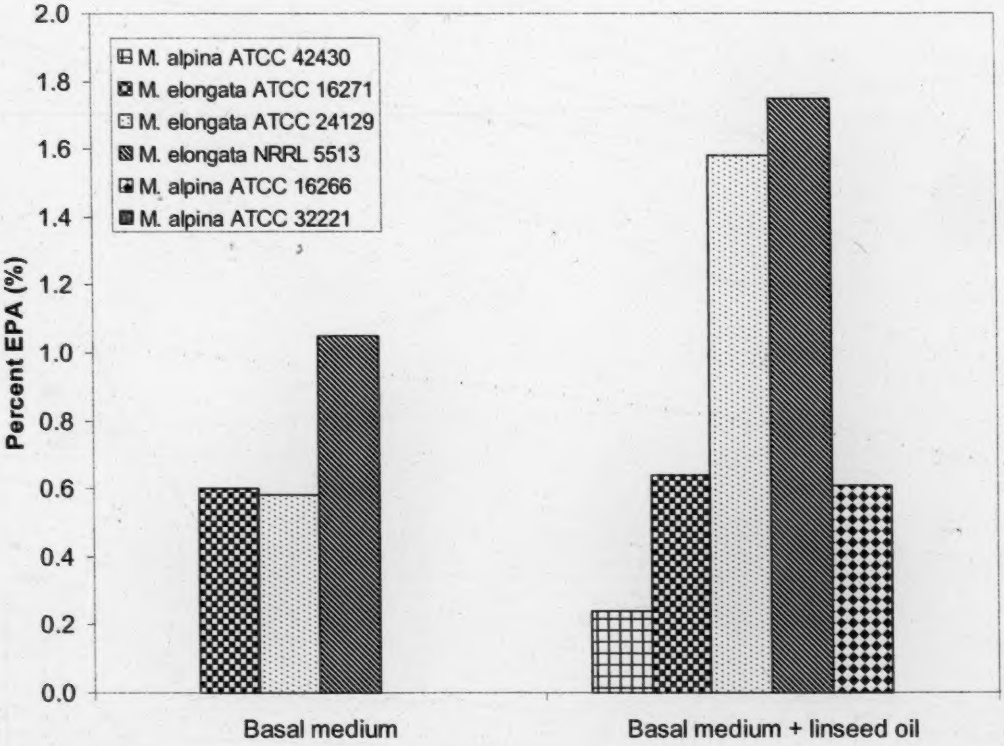

Figure 1-9 Percent EPA for various *Mortierella* strains (11)

appeared to stimulate EPA production in the five *Mortierella* strains that could produce EPA. In fact, the amounts of EPA produced when the moulds were placed at 11 °C were substantially higher than those produced with the linseed oil supplemented media. The *M. elongata* strain which had previously been found to produce the highest amount of EPA was chosen for the final stage of this study. In this stage of the study, the glucose and linseed oil contents of the growth media were varied in conjunction with the growth temperatures in an attempt to examine the effect that these variables had on EPA production. Generally, EPA production increased with linseed oil concentration (thereby decreasing with glucose concentration) whether the moulds were grown at 11 °C or 25 °C. On the other hand, ARA content decreased with increasing linseed oil concentration regardless of growth temperature.

The production of EPA by fungi of the *Mortierella* genus was also studied by Shimizu *et al.* (31). The first phase of this study also involved the effect of linseed oil containing media on various *Mortierella* fungi when they were grown at 12°C. Generally, there was an increase in mycelial mass and EPA content with the addition of linseed oil, though the effect on the ARA content of each strain was variable. This study also examined the effect of linseed oil supplementation to the growth media at two temperatures (12 °C and 28 °C), however, a *M. alpina* strain was used as the mould of interest. When comparing the effect of growing the moulds on linseed oil supplemented media at 12 °C and 28 °C, there are obvious increases in the mycelial yields (mg/mL), as well as, the EPA and ARA contents (mg/mL) when the moulds are grown at 12 °C. At 12°C linseed oil supplementation gives significantly higher mycelial and EPA yields though, for *M. alpina* 20-17, the ARA content is markedly reduced (Figure 1-10).

In addition to linseed oil, some researchers have tested various other growth media supplements in order to examine their effects on the fatty acid compositions of various moulds. One such study was undertaken by Kendrick and Ratledge (21) in which triolein, sesame oil, safflower oil, linseed oil as well as oil extracted from *M. isabellina* were each supplemented to glucose containing media. Four fungi were studied, including a strain of *M. isabellina* and the lipid yields are shown in Figure 1-11. It is interesting to note that *M. isabellina* did not grow on either the linseed oil or *Mortierella* oil containing media. However, the safflower oil supplemented medium resulted in the highest cell yield while the sesame oil supplemented medium actually resulted in a markedly lower cell yield than the glucose medium alone. However, with regards to lipid content of *M. isabellina*, the safflower oil and sesame oil containing media both had the highest yields (more than two times higher than that obtained with the glucose media alone). When taking into account the previously noted cell yield, results indicated that safflower oil was a better supplement for overall lipid yield.

With respect to cultivating *M. isabellina* in order to obtain specific fatty acid molecules, the results are complicated. For example, if a researcher is interested in GLA production, there are two possible choices for growth media composition. On one hand, safflower oil supplementation to a glucose medium results in higher cell and lipid yields than the glucose medium on its own. However, the percentage of GLA that is obtained from the glucose medium without supplementation is more than two times that obtained from the supplemented medium. Considering the fact that the safflower oil containing medium yields more than twice the overall lipid content than glucose does but glucose results in more than twice the amount of the target fatty acid, it is not obvious which of

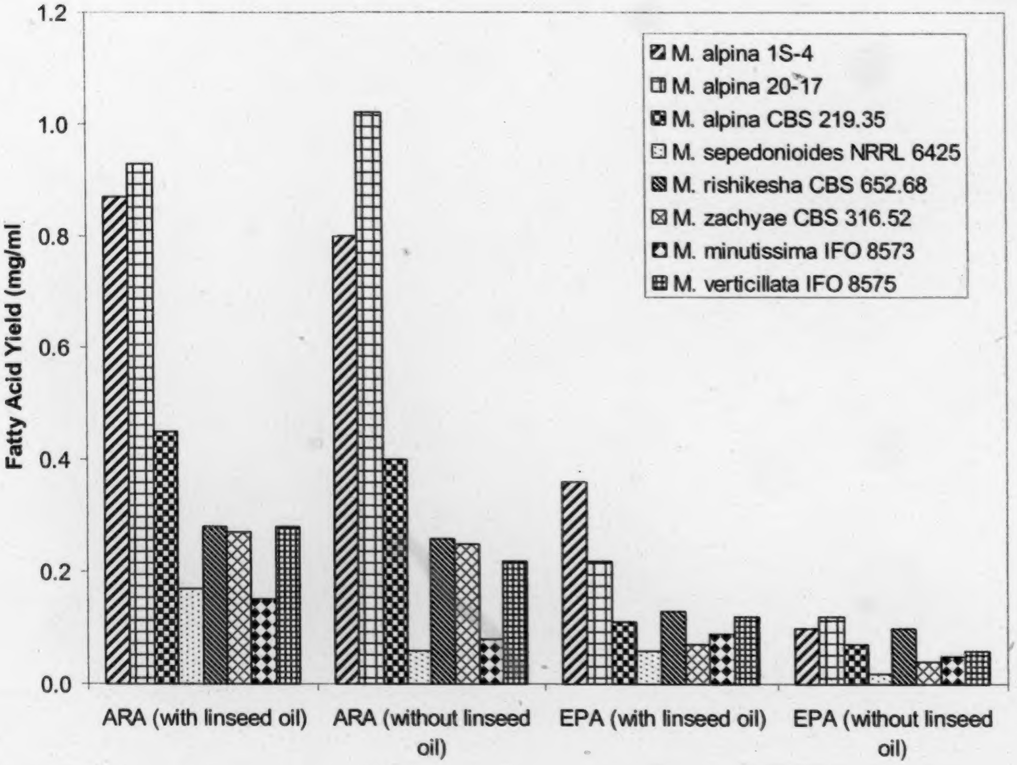

Figure 1-10: ARA and EPA yields for various *Mortierella* strains with and without linseed oil supplementation of the medium (32)

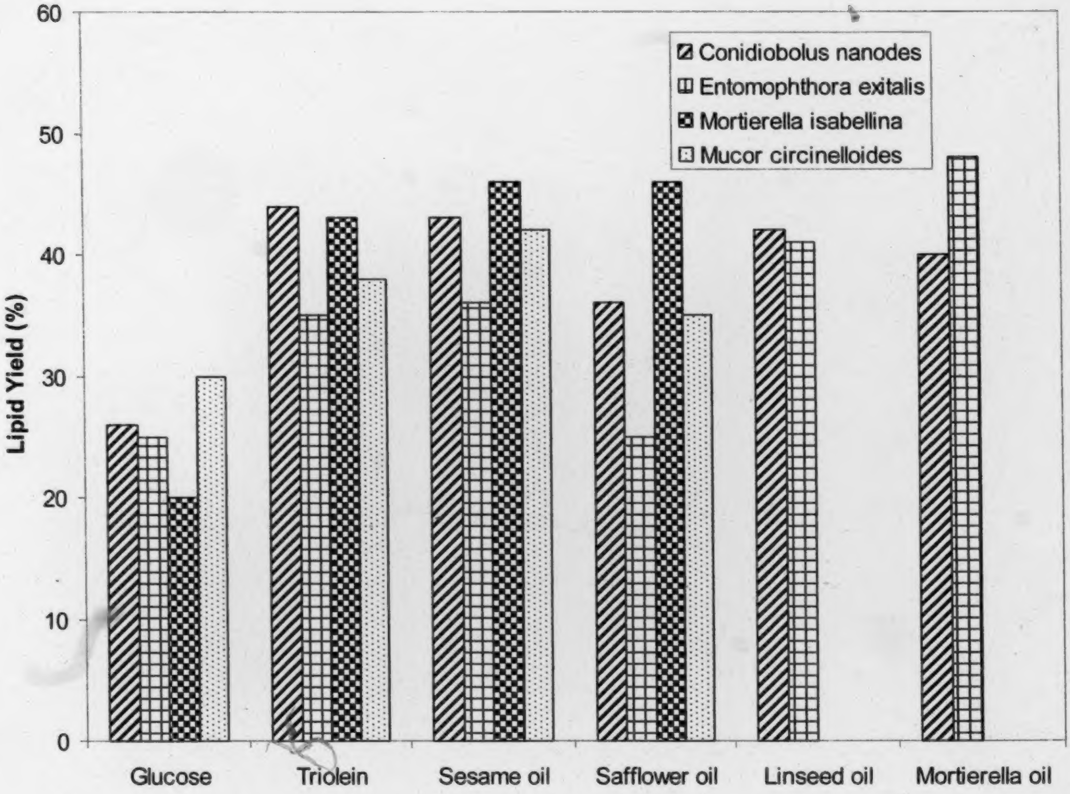

Figure 1-11: Percent lipid yield for various fungal species grown on media containing various carbon sources (21)

these choices, if either, is the better one. Furthermore, it was observed that although DHGLA is present in measurable amounts when *M. isabellina* was grown on the unsupplemented glucose medium, these DHGLA contents dropped to trace amounts when the media were supplemented with any of the oils.

Shimizu *et al.* (28) tested various oils with the express purpose of looking for an increase in DHGLA production in the *M. alpina* 1S-4 strain. Only sesame oil resulted in a significant increase in DHGLA and therefore it was chosen as the supplement with which to use in further investigations of this fatty acid. Several factors were examined in conjunction with sesame oil supplementation of the medium. First, it was observed that the glucose and fatty acids of sesame oil present in the growth medium were almost completely depleted within the first three days of cultivation. Also, the DHGLA/ARA ratio was relatively high when compared to the mould grown on the unsupplemented medium. Furthermore, the DHGLA/ARA ratio of the mould grown in the supplemented medium remained fairly constant throughout the incubation period in contrast to that of the mould grown in the unsupplemented medium which decreased dramatically over time. The second factor that was examined was the effect of various concentrations of sesame oil on DHGLA production. Most notably was the fact that as sesame oil concentration increased, the amount of DHGLA increased while the ARA content decreased. However, the cumulative amounts of ARA and DHGLA remained consistent. The third variable that was explored was the effect of glucose concentration of DHGLA production. It was found that, as with sesame oil concentration, the amount of DHGLA increased with glucose concentration, however, this only occurred until the glucose concentration reached 5%. Further increases in glucose concentration hampered DHGLA production. Nonetheless, the DHGLA/ARA ratio remained fairly constant regardless of the glucose concentration. The fourth component that was examined was the effect of supplementation time of the sesame oil on the production of DHGLA. It was found that there was a higher DHGLA yield when the sesame oil was added within the first three days of cultivation than when it was added later in the growth period.

In order to test whether the fatty acid composition of the sesame oil was the mitigating factor in DHGLA production, sesame oil was mixed with other oils in order to change its fatty acid composition. Results indicated that the fatty acid composition of sesame oil was, in fact, not the determining factor in DHGLA production. This implied that it was the non-oil fraction that was important in increasing the amount of DHGLA production. Addition of increasing concentrations of the non-oil fraction to the *M. alpina* growth medium did, in fact, increase DHGLA production whereas addition of the oil fraction did not. It was suggested in this paper that the reason for the non-oil fraction being the active component of the sesame oil was that it contained an inhibitor that repressed the conversion of DHGLA to ARA (Figure 1-2), thereby effectively increasing the DHGLA content of the fungus. A subsequent study (29) determined that the sesamin contained in sesame oil inhibited Δ5-desaturase thereby preventing the conversion of DHGLA to ARA. Δ5-desaturase is responsible for this conversion therefore it is logical that inhibition of this enzyme would result in an increase in DHGLA yield while causing a decrease in ARA yield.

The ability of different fungi, including *Mortierella spinosa*, to utilize a fatty acid mixture (added to the liquid growth medium) to form GLA was examined in a study by Funtikova and Mysyakina (13). The various fungi were found to utilize the free fatty acids with varying degrees of efficiency. However, the *Mortierella* fungus effectively formed GLA, as well as, ARA and EPA from the mixture. Kavadia *et al.* (18) also focused on the effect that growth media had on GLA production in various fungi, including strains from the *M. ramanniana* and *M. isabellina* species. The cultures were each incubated in a liquid growth medium containing glucose and yeast extract with a pH of approximately 6.0 and at a temperature of approximately 28 °C. The *Mortierella* fungal strains accumulated significant amounts of lipids after the nitrogen source was exhausted. However, once the glucose was depleted from the media, the lipids were consumed. Presumably, the lipids were being used as an alternate carbon source for further mould growth. The GLA content, which was relatively low during lipid accumulation, increased as the lipids were degraded. This implies that the conditions which are conducive to GLA production are those which favor mould growth rather than lipid accumulation. The fact that GLA was found to be produced during fungal proliferation supports this theory.

The Funtikova (13) study provides experimental evidence to support the two-phase growth theory of oleaginous fungi. Although logic dictates that the most favourable conditions for the production of a given target fatty acid would be those conditions which favour lipid accumulation, interestingly the results of the Funtikova study have shown that this is not necessarily the case. However, these same results also support the supposition that the composition of the growth media is a pivotal factor in manipulating the fatty acid composition of fungal oils.

In order to further increase the ARA yield of *M. alpina* 1S-4, Shinmen *et al.* (33) tested various supplements to glucose/yeast extract growth media including fatty acids, *n*-

alkanes, and natural oils. Results showed that ARA production increased 2.8 times when soybean oil was used as a supplement when compared to unsupplemented media. Corn, olive, rapeseed, and fish oils also increased ARA production. Stimulatory effects were also observed when either olive oil or cottonseed oil was used as a carbon source and yeast extract was used as the nitrogen source. Another important observation was that all of the oils tested effectively stimulated ARA production once the growth media were additionally supplemented with significant amounts of oleic and linoleic acids.

These experiments showed that, like carbon and nitrogen sources, optimization with various oils and fatty acid mixtures gave variable results across the fungal species tested. The majority of the studies focused on the use of various seed oils containing relatively high concentrations of LA and ALA as precursors for DHGLA, ARA, and/or EPA. The presence of LA and ALA in the oils used for media supplementation seems to be effectively utilized by the various fungi. Presumably, this allows the fungi to reserve its carbon source for functions other than EFA production instead of synthesizing them *de nova*.

Distribution of EFAs Within Fungal TAG Molecules:

The relative partitioning of various fatty acids of interest among lipid fractions is not the only distribution that researchers must take into account when studying the lipid profiles of potential EFA sources. The positional distribution of EFAs within TAG molecules is also important since it is the limiting factor with respect to the bioavailability of the EFAs for entrance into the ω-3 and ω-6 pathways and prostaglandin production. Although most studies reported the fatty acid composition of various oils containing target fatty acid molecules, this does not take into account the composition of the TAG molecules. Regardless of the content of a given EFA within a particular oil, if the fatty acid in question cannot be efficiently utilized by the human body, then it is an unsuitable source of that EFA.

Myher *et al.* (54) examined the positional distribution in addition to the fatty acid compositions of various oils, including three oils obtained by *M. alpina*. They found that, in the *Mortierella* oils, there was preferential positioning of EPA at the *sn*-1 and *sn*-3 positions of the TAG molecules while ARA was found primarily at the *sn*-1 position. On the other hand, GLA and DHGLA were preferentially positioned at the *sn*-2 position. With regards to cleavage by pancreatic lipase, these positionings imply that GLA and DHGLA have lower bioavailabilities for utilization within the ω-3 and ω-6 pathways within these oils when compared to EPA and ARA. Another study involving the positional distribution of ARA within TAG molecules found in oils extracted from *M. alpina* was performed by Liu *et al.* (56). The fungal oils were obtained from four suppliers and analyzed. It was found that there were quantitative differences between these oils with respect to their fatty acid compositions as well as the positional distributions of ARA within their respective TAG molecules.

The implications of these findings are two-fold. First, it shows that the same type of oil obtained from different suppliers may not consist of the same amounts of a given fatty acid. Secondly, these results also imply that the bioavailability of the fatty acids present in these oils is not necessarily consistent within each type of oil if it is obtained form different suppliers. For example, two oils may have the same fatty acid composition but their fatty acids may be arranged differently within the TAG molecules.

Therefore, the bioavailability of a given fatty acid may not be the same between any two oils.

Industrial Production of EFAs from Fungal Sources:

The differences that Liu *et al.* (56) found in the bioavailability of ARA in fungal oils from different suppliers raise an interesting point. The industrial production of any EFA by a fungal source must be organized in such a way as to ensure that EFAs are being effectively and reproducibly produced. This is no easy task given the multitude of variables that must be regulated. In addition to the complexities that plague fungal research at the laboratory level, another level of difficulties arises when these productions are scaled up in an attempt to simulate industrial operations.

A few studies have been performed in which researchers have attempted to scale up their fungal productions. It is generally agreed that while solid state cultivation yields higher EFA production in *Mortierella* species, submerged fermentations are easier to scale up and more feasible for industrial production (6, 14, 22, 33, 35, 37, 38). In a study by Stredanska *et al.* (34), *M. alpina* was grown on a number of solid substrates in an attempt to maximize ARA production on an industrial scale. It was found that millet and barley gave the highest ARA yields when compared to rice and wheat substrates. However, further experimentation indicated that there was an inverse relationship between scale and ARA yields, though millet performed consistently better than barley in these scale-up experiments.

Lai *et al.* (22) also performed a study with *M. alpina* where solid state fermentation was compared to submerged cultivation with respect to DHGLA production. The submerged culture media consisted of a basal medium composed primarily of glucose and yeast extract which was then supplemented with either sesame oil or the non-oil fraction isolated from sesame seeds. The solid state culture was composed of milled sesame seeds moistened with water. The decision to use sesame seeds and their components in this study was based on results from previous studies which indicated that the non-oil fraction of sesame oil inhibited the conversion of DHGLA to ARA thereby increasing the yield of DHGLA (28, 29). The Lai study found that solid state cultivation yielded nine times the DHGLA than either of the submerged cultures, which supports the general consensus that solid state cultivation yields higher EFA productivity than submerged cultivation. However these comparisons were not made on large scale cultures and, as other studies have shown (6, 14, 25, 33, 37, 38), industrial size scale-ups present problems not encountered in smaller scale experiments.

A study was carried out by Hansson and Dostalek (14) on four *Mortierella* strains (two *M. ramanianna* strains, *M. vinacea*, and *M. isabellina*) using submerged cultures. A number of different large-scale reactor systems were employed in an attempt to scale up production of these mould species, however, growth was poor for each of the strains tested. It was suggested that the poor growth within these large-scale systems was due to the susceptibility of these fungal strains to physical stress. A scale up study by Yokochi *et al.* (38) also encountered the problem of poor fungal growth when its production was moved from a flask culture to a stirred-tank fermentor. The cause of this poor growth was reasoned to be due to the loss of decane from the culture medium. Therefore, decane was continuously fed into the culture solution and the cultivation temperature was lowered in an attempt to prevent this loss from occurring. At seven days of cultivation, the dry cell weight was almost three times higher than it had been in the shaker flask studies and the total lipid content was almost four times what it had been in the shaker flasks.

Another scale-up study was performed by Totani *et al.* (37). In this study, the focus was on the scaled-up production of ARA by *M. alpina*. Since liquid cultivation is easier to carry out than cultivation on solid media, it is preferable to use liquid media when producing fungal oils on an industrial scale. However, fungal growth in liquid media tends to produce modest lipid yields with relatively low ARA contents. Therefore, the ideal situation for industrial production of this fatty acid with *M. alpina* would be to optimize the growth conditions in such a way as to increase the mycelial and ARA yields to at least the level that they are in a solid medium by using a liquid medium. In an attempt to achieve this goal, Totani *et al.* (37) scaled up their *M. alpina* production in a stepwise manner, thereby allowing for the necessary adjustments to be made as the production size increased. They began with shake flask studies using 200 mL of media to determine the effect of varying dextrose concentration at this level of fungal production before moving up to the bench-top fermentations using 4 L of media. At the level of bench-top fermentations, aeration rate was added as an additional variable. In the shake flask studies, various percentages of dextrose ranging from 5% to 25% were tested for their effects on fungal growth and ARA content. The moulds were cultivated at 20 °C with a shaking speed of 120 rpm for 20 days. Results at this level of production indicated that 5% dextrose concentration yielded the highest ARA content although the cells grew more slowly than those grown in media containing up to 20% dextrose. When the dextrose concentration in the media exceeded 20%, cell growth was repressed.

At the bench-top fermentation level, 10% dextrose was used in the media and the moulds were incubated for 16 days at 20 °C. The agitation speed was set at 100, 300, or 500 rpm for a given sample. Results from this portion of the study indicated that the optimum conditions for ARA production at this scale was high dextrose concentration coupled with high agitation speed. These optimum conditions were tested on a pre-pilot scale before moving onto the pilot scale production. It was found that the optimum conditions outlined at the bench-top fermentation level was easily scaled-up to the level of the pilot scale fermentation. The researchers of this study suggested that an initial growth period with high dextrose concentrations followed by a period of starvation may be the ideal method of inducing high ARA production since the ARA content was found to greatly increase after dextrose exhaustion of the medium.

A similar study was carried out by another research group (6, 33) who also used *M. alpina* as the mould of interest. Based on initial experiments, it was concluded that the optimal conditions for the growth of *M. alpina* on a large-scale production (i.e., using a 2000 L fermentor) was to use a liquid medium composed of 2% glucose, 1% yeast extract, and 0.2% olive oil and adjusted to pH 6.0. The moulds were incubated at 28 °C for 10 days with an aeration rate of 1 vvm (volume per volume per minute) and an agitation rate of 80 rpm. The mycelia were then harvested and allowed to stand for another few days. While the palmitic, stearic, oleic, and linoleic acids decreased upon standing, the ARA content remained the same.

A study by Lindberg and Molin (25) looked at the effect that temperature and glucose supply had on EFA production by *M. alpina* when grown in large-scale cultures. The optimum pH for ARA and lipid production was pH 6.5 at 25 °C while ARA production was minimized at 12 °C. Both EPA and GLA were maximized at 12 °C. Lipid production was optimized with an excess of glucose and a nitrogen-limited environment. ARA yield was maximized with glucose exhaustion.

Genetic Mutants:

One major advantage to utilizing *Mortierella* and other fungi in the industrial production of EFAs is the ability to manipulate the fatty acid profiles of these organisms without the requirement of genetic manipulation. However, some research has been performed using Δ5-desaturase-defective mutants of *Mortierella alpina* 1S-4 as potential industrial producers of DHGLA. These mutants lack the ability to efficiently convert DHGLA to ARA, though not all are completely deficient in Δ5-desaturase activity. Jareonkitmongkol *et al.* (17) reported on the isolation and characterization of three desaturase-defective *M. alpina* 1S-4 mutants. These mutants were designated Mut44, Mut49, and Mut48 and were deduced to be Δ5-, Δ6-, and Δ12-desaturase-defective, respectively. Mut44 yielded a significantly high percentage of DHGLA (28.6% w/w) and a relatively low ARA yield (10.6%) when compared to its wild type (6.3% and 47.0%, respectively). This trend is comparable to that which has been observed with the addition of sesame oil or sesamin to the growth media (28, 29). In these previous studies it has been reported that the non-oil fraction of sesame oil is responsible for the suppression of Δ5-desaturase activity which converts DHGLA to ARA. LA made up 46% of the total fatty acids in Mut49 whereas the DHGLA and ARA contents were relatively low when compared to the wild type. The conversion of LA to GLA (which is the immediate precursor to DHGLA) is mediated by Δ6-desaturase, therefore, the accumulation of LA coupled with the low productivity of DHGLA and ARA indicate that Δ6-desaturase activity has been inhibited in this mutant. Mut48 yielded no LA, DHGLA, or ARA production in the basal medium. However, in media supplemented with LA, ARA was produced which indicates that this mutant was only defective in its Δ12-desaturase.

More intensive experiments were performed using Mut44 in order to determine the effects that various factors including glucose concentration, yeast extract concentration, growth temperature, media supplementation, aging, and scale-up had on its DHGLA production (15, 16). Results indicated that, although the highest concentration of DHGLA was obtained with 4% glucose, the highest absolute DHGLA yield was obtained at a glucose concentration of 6%. An increase in glucose concentration beyond 6% resulted in a decrease in DHGLA yield. Similarly, the maximum DHGLA yield for yeast extract with 2% glucose was 0.5%. Higher concentrations of yeast extract resulted in decreased DHGLA yield. Though fungal biomass yield increased with increasing temperature, DHGLA content decreased with increasing growth temperature. ARA yield was not significantly different between the temperatures tested. The addition of various oils to the basal medium resulted in increased biomass yield and decreased DHGLA production though the overall DHGLA yield increased with the addition of these oils. The highest DHGLA yield was obtained with the addition of sesame oil which indicates that Mut44 may not be completely deficient in Δ5-desaturase activity. Previous studies have found that the wild type *M. alpina* 1S-4 increased its ARA yield upon aging of its harvested mycelia (33). Although a similar trend was observed for both ARA and DHGLA yields for Mut44, the increase in yield during aging of the harvested mycelia was more significant for ARA (from 13 mg/g dry biomass to 23 mg/g dry biomass) than for DHGLA (from 51 mg/g dry biomass to 58 mg/g dry biomass). At the level of benchscale fermentation it was found that glucose feeding of the media was required for effective DHGLA production.

Kawashima *et al.* (19) studied DHGLA production in another *M. alpina* 1S-4 Δ5-desaturase-defective mutant, *M. alpina* S14, on an industrial scale-up level. Soy flour was used as the nitrogen source and glucose was the carbon source. The temperature used for cultivation was 26 °C since DHGLA production was higher at this temperature when compared to 28 °C. At 12 days, DHGLA content was 7.0 g/L of culture medium which amounted to 43.9% of the total fatty acid content.

Cloning and Expression of Δ-Desaturases from *Mortierella*:

In addition to studying Δ-desaturase mutants of *M. alpina*, research has also been done in the area of cloning specific Δ-desaturases from various *M. alpina* strains and expressing them in a number of other organisms. Research has shown that EFA production in *Mortierella* spp. can be effectively manipulated by simply varying the growth conditions and without genetic intervention. However, there is also promising research that shows that desaturase genes cloned from *Mortierella* spp. can be utilized in the transformation of other organisms, thereby making them potential EFA producers. This body of research includes the cloning of Δ5-, Δ6-, Δ9-, and Δ12-desaturases from *M. alpina* and expressing them in *Aspergillus oryzae*, *Saccharomyces cerevisiae*, and canola seeds.

Sakuradani *et al.* (57) cloned Δ9-desaturase from *M. alpina* 1S-4 and expressed it in *Aspergillus oryzae*. This particular enzyme is responsible for the desaturation of PA to POA and SA to OA. The expression of the *Mortierella* Δ9-desaturase gene in *A. oryzae* resulted in more than doubling the contents of POA and OA with corresponding decreases in the PA and SA contents. A second study by Sakuradani *et al.* (5859) cloned the Δ12-desaturase gene from *M. alpina* 1S-4 and expressed it in both *A. oryzae* and *S. cerevisiae*. The Δ12-desaturase gene is responsible for the conversion of OA to LA. The LA content in the fatty acid profiles for both of the transformed organisms increased over that which was present for their control counterparts (from 0.0% to 8.5% for *S. cerevisiae* and from 58.1% to 71.9% in *A. oryzae*).

Huang *et al.* (59) cloned the Δ12- and Δ6-desaturase genes from an *M. alpina* strain and expressed them both in *S. cerevisiae*. The Δ6-desaturase converts LA to GLA and ALA to stearidonic acid (SDA). The expression of the Δ12-desaturase gene alone resulted in an increased LA content while the expression of the Δ6-desaturase gene by itself showed increased SDA and GLA contents (when the appropriate fatty acids were added to the substrates). In addition, co-expression of these enzymes resulted in *de nova* synthesis of GLA within the yeast (i.e., endogenous OA was converted to LA by the Δ12-desaturase and the LA was then converted to GLA via the Δ6-desaturase).

Another study looked at the cloning of a Δ5-desaturase gene from *M. alpina* and expressing it in *S. cerevisiae* (60). The transformed yeast was able to accumulate ARA when DHGLA was added to the substrate. Similar results were obtained from another study which expressed a Δ5-desaturase isolated from *M. alpina* and transformed into *S. cerevisiae* (61). In addition, the Knutzon (61) study expressed this enzyme in a low linolenic acid variety of *Brassica napus*. Since, as shown in Figure 1-2, GLA is a precursor to DHGLA, there was no DHGLA present in the canola and therefore no ARA was produced in the presence of the Δ5-desaturase. However, both taxoleic and pinoleic acids (which are Δ5-desaturation products of OA and LA, respectively) were present in the canola seeds.

Conclusions:

Though there is still a long way to go before some *Mortierella* fungi can be used as major sources of EFAs on an industrial scale, *Mortierella alpina* is already being utilized as an industrial source of ARA. The industrial production of other EFAs using *Mortierella* species appears to be the next step in utilizing this fungal genus as an alternate source for EFAs. However, there are many variables that must be standardized before this can happen, including deciding which fungal strains should be used for the production of each specific EFA. In the case of ARA and EPA, it appears that the fungal production of one may have to be sacrificed in order to produce the other, however, it may also be possible to use one mould strain for the production of various EFAs. For example, there is an EPA/ARA ratio within *Mortierella* fungi that can be effectively manipulated according to consumer demands.

There also needs to be more studies involving the systematic manipulation of growth variables for *Mortierella* strains that have the potential to become industrial EFA producers. This is necessary in order to effectively evaluate the effects that each variable has on EFA production and for efficient optimization of scale-up productions. Furthermore, there needs to be more research done on the TAG profiles of the various mould strains which are potential industrial EFA producers in order to assess the bioavailability of these strains with regards to the EFAs they are to be used for. A potential EFA producer is only effective if the EFAs it produces can be efficiently utilized by the human body for entrance into the ω-3 and ω-6 pathways and/or prostaglandin production and if the EFA profile can be reliably reproduced. However, regardless of the obstacles that must still be overcome in this area of research, the use of *Mortierella* fungi as potential industrial producers of EFAs is still a very promising endeavor.

The objective of this Masters project was to systematically determine the effects that pH, temperature, carbon source, nitrogen source, metal ions, and oil supplementation of the media had on the growth, lipid accumulation, and GLA production on a locally isolated strain of *M. ramanniana* var, *ramanniana*. Since no previous research has been published on this strain, it is important to investigate these media effects in this particular organism. Previous research has shown that, among *Mortierella* spp., media variables can have diverse effects on different strains within a species (i.e., the same trends are not necessarily observed for a given variable across all strains). Therefore, one cannot necessarily deduce the way in which a given *M. ramanniana* strain will react to a given culture condition based on studies done using other strains. It is for this reason that every novel strain must be studied independently.

In this project, the aforementioned variables were manipulated such that each variable was independently optimized prior to the optimization of its successive variable. The exception to this succession was the last two experiments in Chapter 3 (metal ion addition and oil supplementation of the medium) which were independent of each other and which both used the same basal medium based on the first four experiments.. The ultimate objective of these experiments was to determine whether this particular *M. ramanniana* strain could be a potential industrial producer of GLA once the above variables have been optimized.

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Chapter 2

Maximizing the Production of *Gamma*-Linolenic Acid in *Mortierella ramanniana* var. *ramanniana* as a Function of pH, Temperature and Carbon Source

Introduction:

In the current health climate, consumers have become very aware of the effect that different types of fats have on their health. It is generally accepted that *omega*-3 and *omega*-6 fatty acids are vital for good health (1). These essential fatty acids (EFAs) are essential to human health, but are not synthesized by the human body. As such, a healthy diet needs to include these fundamental components. Since the public has become increasingly aware of the importance of these omega fatty acids, the market has become primed for increased production of foods containing EFAs. As a result, industrial producers are attempting to provide consumers with novel dietary options that incorporate EFAs into their diets. One such example is an egg-substitute product fortified with omega fatty acids that was introduced on the market a few years ago and is still being advertised as a "heart healthy alternative" (e.g., "Omega-egg", University of Nebraska). Another example is an infant formula enriched with arachidonic acid (ARA) and docosahexaenoic acid (DHA) (an *omega*-6 and *omega*-3 fatty acid, respectively) that has been introduced to the market by various companies (e.g., Mead Johnson and Co.).

The introduction of foods enriched with EFAs requires an increase in the industrial production of these fatty acids. Since the traditional sources of EFAs used by industry offer relatively low yields at relatively high costs, some researchers have been attempting to find alternate, relatively inexpensive sources that produce higher yields.

Currently, the main industrial sources of EFAs are fish oils, although other sources are also used. Arachidonic acid, for example, is commercially isolated from animal livers and sardines while eicosapentaenoic acid (EPA) is also extracted from algae and moss (2, 3). Currently, the major source of *gamma*-linolenic acid (GLA) is evening primrose oil (2, 3).

Some of the alternate sources that are currently being researched include fungal species that are known to be oleaginous organisms. Although various plant sources are also being studied as alternatives, fungal sources give researchers the advantage of manipulating the EFA profiles of the organisms by simply varying their growth conditions rather than having to resort to genetic manipulation or extensive breeding efforts. Although the growing conditions of plants can also be varied, the effect that these variations have on the resulting fatty acid profiles are not as substantial as those obtained by the manipulation of fungal growth conditions. Fungi also have the added benefit of not requiring agricultural land for production, which can be a limiting resource. In addition, the plausibility of using a fungal source as an alternative for EFA production has been validated by the fact that oil rich in ARA is being been extracted from *Mortierella alpina* (4 - 11) and utilized in the production of the aforementioned infant formula (e.g., Martek Biosciences Corporation).

One possible alternate source for GLA can be found in the fungal genus *Mortierella*. This genus is further subdivided into two subgenera, *Mortierella* and *Micromucor*. The main difference between these two subgenera is that species in the *Mortierella* subgenus can produce fatty acids up to and including the C20 fatty acids while those in the *Micromucor* subgenus can only produce fatty acids containing a maximum of 18 carbons (C18) (12). Fatty acids up to 18 carbon lengths can be synthesized *de nova* but production of longer chain fatty acids require the action of elongation enzymes (elongases) (13). Presumably, this deficiency in C20 fatty acid production is a result of a mutation or other type of selection that inactivated or deleted the elongase enzymes normally responsible for the production of the longer chain fatty acids. With regards to GLA production, this difference in biochemistry suggests that species found in the subgenus *Micromucor* would be more viable sources of GLA since their fatty acid pathways do not allow for the further elongation of C18 fatty acids. The species *Mortierella ramanniana* var. *ramanniana* belongs to the subgenus *Micromucor* and, as such, may be a viable source of GLA.

GLA is an *omega*-6 fatty acid that is a precursor to other EFAs, such as the aforementioned DHGLA, as well as an eicosanoid precursor. Eicosanoids are a biologically important class of molecules that have hormone-like actions in the body. These actions include inflammatory responses and prevention of blood-clotting responses, in addition to a number of other functions (14). It is these functions that make them vital to the maintenance of homeostasis in the body, including but not limited to those related to the circulatory system. They are also crucial to brain development during the fetal and infant stages of life (1, 14).

Previous research on *Mortierella* and other fungi have shown that a number of media variables can affect the growth and lipid accumulation that occur within various species. These include pH, temperature, carbon source, nitrogen source, carbon/nitrogen (C/N) ratio, metal ion concentration, and supplementation of the media (15 - 27). However, none of these studies consecutively evaluated the effects that each of these variables had on growth, lipid accumulation, or EFA production in a manner where each subsequent growth factor was dependent on a previously tested variable. Moreover, none of these studies have focused specifically on growth media effects on *M. ramanniana* var. *ramanniana* with regards to GLA production. The growth manipulation studies involving *M. ramanianna*, to date, have focused on the var. *angulispora* (26, 27, 30), though there have been studies that have looked at the fatty acid profiles of some *M. ramanniana* var. *ramanniana* isolates (13). This study focused on the first three media variables (pH, temperature, carbon source) and was performed using a locally (Perryvale, Alberta, Canada) isolated strain of *M. ramanniana* var. *ramanniana*. Since the intent of these experiments was to accumulate data which eventually leads to scale-up and production of these moulds for mass GLA production, only broth media were tested since these types of media are more easily scaled-up than solid media (5, 6, 8, 9, 11, 24).

Experimental Procedures:

Experiment 1 was performed to determine the optimum initial pH for growth of a strain of *Mortierella ramanniana* var. *ramanniana* isolated from a sphagnum peat bog near Perryvale, Alberta by Dr. Akihiko Tsuneda. An identified culture was provided by Dr. Tsuneda for this investigation and was maintained in potato-dextrose agar (PDA) (supplied by Difco, Le Pont de Claix, France). Plugs of agar containing fungal mycelia were extracted from the PDA stock cultures using a corker (1 cm inner diameter) and were transferred to fresh petri dishes every ten days to prevent depletion of the media. To ensure that experimental media were inoculated with fungal mycelia of the same age, one plug was placed in the centre of each PDA plate and the plugs used for inoculation of experimental growth media and for fresh stock cultures were taken from the outer rim of growth.

Three different media were used for pH determination in order to compensate for any pH-media interactions. They included cornmeal broth (CMB), potato-dextrose broth (PDB), and dextrose-yeast broth (DYB) media. DYB was chosen because research has shown that it is an excellent growth medium for *Mortierella* spp. (6, 7, 15-17, 19-21, 23, 24). The PDB and CMB media were chosen based on the fact that they were determined to be nutritionally intermediate and poor growth media, respectively, for this particular strain of *M. ramanniana* var. *ramanniana* from previous experiments conducted in our laboratory.

The CMB medium was prepared by adding 50.0 ± 0.3 g of cornmeal to 1.000 ± 0.005 L of deionized water and bringing the mixture to a boil. The temperature was reduced to 60.0 ± 1.2 °C and the mixture was left to simmer for twenty minutes. Once the infusion had cooled to room temperature (22.4 ± 0.3 °C), it was strained through a cheesecloth to remove the cornmeal and the resulting volume was brought back up to 1.000 ± 0.005 L by adding fresh deionized water. The PDB medium was prepared by first peeling and cubing 200.0 ± 1.8 g of red potatoes into approximately 2 cm x 2 cm x 2 cm pieces, then, adding the pieces to 1.000 ± 0.005 L of deionized water. They were then brought to a boil and left to boil for one hour. The infusion was strained and the resulting liquid was brought back to volume by adding fresh deionized water. Then 20.0 ± 0.2 g of dextrose was added to the infusion broth. The DYB medium was prepared by simply adding 20.0 ± 0.2 g of dextrose and 10.0 ± 0.2 g of yeast extract to 1.000 ± 0.005 L of deionized water.

The resulting media were each transferred to 250 ml Erlenmeyer flasks (50 ± 2 ml/flask) and then autoclaved for 25 minutes at 121 °C. Once the media had reached room temperature (22.4 ± 0.3 °C), the pH's were adjusted in one degree increments from pH 1.00 to pH 14.00 (all pH's were accurate within 0.05 units) by the addition of 1.0 M solutions of NaOH and HCl. These solutions had also been autoclaved to decrease the risk of contamination. Once the pH's had been adjusted, the media were inoculated by one plug each of stock culture, covered, placed in an orbital shaker set to 100 rpm, and incubated at room temperature for 10 days. Random samples were run from each set of media at different pH's using 1.0 M solutions of tartaric acid and potassium hydroxide in order to eliminate the uncertainty of whether the effects that were seen on fungal growth were actually due to initial pH of the media rather than interaction effects of the solutions used to adjust the pH.

After ten days of growth, the mycelia were filtered using No.1 grade filter paper and left to dry in a 60.0 ± 0.5 °C oven for 24 hours or until no observable decrease in mass was observed. The resulting dry biomasses were recorded for each condition (to 0.0001 g).

Experiment 2 was performed in order to determine the temperature range of growth for this *M. ramanniana* strain, as well as to determine what effect temperature has on lipid accumulation in this fungus. Another objective of this experiment was to determine whether there were any pH-temperature interactions. The same media that were used in the previous experiment were again used for this experiment and were prepared as outlined in experiment 1. Each medium was adjusted to three pH's; 3, 6, and 9. These represent the range of initial pH's within which this *M. ramanniana* strain grows well for all media used at room temperature, as determined by the results obtained from experiment 1. The temperatures tested were 0 °C, 10 °C, 20 °C, 30 °C, and 40 °C (all temperatures were accurate to ± 0.5 °C). The lowest and highest temperatures in this range were chosen as the end points for temperature testing since no growth occurred in any of the media conditions at either of these temperatures.

Once the media were inoculated, they were placed on orbital shakers set to 100 rpm and placed in temperature-controlled chambers set to the appropriate temperature. The cultures were left to grow for ten days, filtered, and allowed to dry as before. The mycelia were ground with a mortar and pestle placed in No. 1 filter paper and attached to a GoldFisch apparatus. Petroleum ether was then refluxed through the samples for 4 hours and a blank sample was run with each set of samples to measure any residue that was left by the solvent itself. Once the runs were completed, the majority of the solvent was boiled off and the remaining solvent was allowed to evaporate off at room temperature overnight. The samples were then measured at least twice during the following day to ensure that there was no observable change in the mass recorded (i.e., to ensure that the solvent had completely evaporated). The resulting lipid yields were then recorded and any solvent residue that had been recorded from the blank run was subtracted from the final results.

Experiment 3 was performed to determine the optimum carbon sources and concentrations for growth, lipid yield, fatty acid profile and absolute GLA content. The results from the previous two experiments suggested that this organism is relatively pH insensitive and accumulates the most lipid at 20 °C, regardless of media used. Since 20 °C is relatively close to room temperature (22.4 ± 0.3 °C) and the ultimate goal of this project is to optimize the growth conditions for industrial GLA production, the remaining experiments were performed at room temperature. Also, the pH's of the media were not adjusted to a specific pH. However, the initial pH's were measured prior to inoculation to ensure that the natural pH's of the media fell within the allowable growth ranges obtained from experiment 1 (average initial pH's for each set of replicates shown in Table 2-1). All the replicates within a given media condition fell within 0.1 pH units of the values shown in Table 2-1.

One percent yeast extract (w/v) was used as the nitrogen source for all the media except PDB which is a complete medium in and of itself. A number of carbon sources were tested, including dextrose, fructose, glycerol, malt extract, potato-dextrose, sucrose, beet pulp and sodium acetate each at concentrations of 1%, 2%, 5%, 10%, 15%, and 20% (w/v of the effective carbon content).

As before, the cultures were allowed to grow for ten days, filtered, dried, and their lipids were extracted. In addition to the dry biomasses and lipid yields, the fatty acid profiles were also recorded for the remaining experiments. Fatty acid methylations were performed using 10:1:1 methanol:chloroform: HCl as per the procedure outlined by Lewis *et al.* (30). The FAMEs were analysed using a Varian 3400 GC equipped with an FID detector set to 240 °C and a BPX70 column (120 m). The injector temperature was set to 180 °C and the column was run using an isothermal program at 180 °C. All experimental samples for the above three experiments were replicated in quadruplicate.

Results and Discussion:

Previous studies that have looked at the effect of fungal growth conditions on lipid accumulation and EFA production in *Mortierella* spp. have used maximum

Table 2-1: Measured pH values of the media used in experiment 3.

Carbon (%)	DYB	FYB	GYB	PDB	SYB	SAYB	MEYB
1	5.52	5.28	5.58	4.69	5.61	6.36	5.58
2	5.33	5.19	5.59	4.69	5.76	6.49	5.43
5	5.20	4.95	5.61	4.59	5.56	6.70	5.50
10	5.11	4.85	5.63	4.59	5.51	6.81	5.32
15	4.92	4.77	5.60	4.56	5.52	6.79	5.03
20	4.99	4.74	5.62	4.54	5.45	6.73	4.99

incubation periods ranging from 4 to 14 days (4 - 11, 15 - 29). An incubation period of ten days was chosen for this study to ensure that any medium that may have resulted in initially slow growth followed by a rapid succession of growth would not be mistaken as a poor growth medium (as it may have been with a four day growth period). On the other hand, time in excess of ten days would have yielded no further benefits as sufficient growth would have occurred within any potentially successful growth media by this time (5, 6, 18-22, 29).

In experiment 1, the pH's of three different growth media were manipulated to determine the pH range of growth for this organism. As shown in Figure 2-1, there were no significant differences between the dry biomasses obtained per medium as a function of pH between pH 3.0 and 10.0 (all biomasses within a specific medium fell within the calculated error ranges). Although there was growth at pH 2.0 for all of the media, the dry biomass yields were between 23.5% and 63.1% of those obtained at the higher pH's within a given medium. Similar results were obtained in the random samples whose pH's were adjusted using tartaric acid and potassium hydroxide (i.e., all results obtained from those samples fell within the error ranges of equivalent experimental pH samples). It is also interesting to note that although there was growth at pH 11.0 in the PDB growth medium, the maximum pH where growth was observed for the other two media was 10.0. There was no growth observed in any of the media at pH's 1.0, 12.0, 13.0, or 14.0.

Previous experiments that have examined pH as a growth variable for this fungal species have focused on the pH range from 4 to 9 (5, 22, 29). A study by Lindberg and Molin (5) found that *M. alpina* did not grow at pH 8.5 and that the biomasses were lower

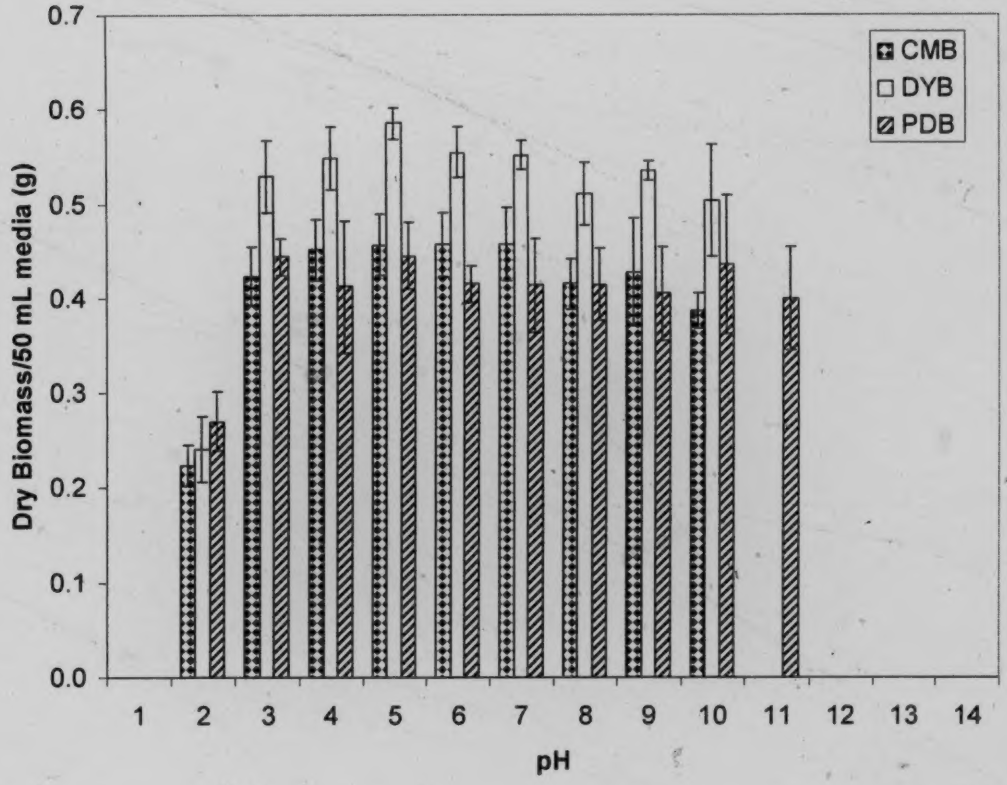


Figure 2-1: Dry biomasses per 50 ml of media at different pH's at room temperature. (CMB = cornmeal infusion broth; DYB = dextrose yeast broth; PDB = potato-dextrose broth)

at pH 7.5 compared to those recorded at pH's 5.5 and 6.5. Xian *et al.* (29) observed an inverse relationship between dry biomass and pH for *M. isabellina* between pH 5 and 9.

In the current experiment, results from experiment 1 (Figure 2-1) indicated that a locally isolated strain of *Mortierella ramanniana* var. *ramanniana* is relatively pH insensitive (between pH 3.0 and 10.0) as there were no significant deviations in growth within this pH range for any of the media tested. However, a major media-pH interaction was evident at pH 11.0. Neither PDB nor CMB contained any yeast extract as a nitrogen source since they are considered complete media in and of themselves. However, both PDB and DYB contained dextrose as part of their composition. Yet, neither CMB nor DYB grew at pH 11.0 whereas PDB did. The major difference between PDB and the other two media used in this experiment is the presence of potato starch. Also, since this medium was prepared directly from a potato infusion broth, it may contain other components that may have affected its growth. Since enzymes need specific conditions under which to effectively work (including pH and, sometimes, certain cofactors), it is possible that some factor within the potato infusion broth itself enhanced its growth at pH 11.0. The results from this experiment imply that there are some media-pH interactions that occur which can affect the growth of this particular organism, and this warrants further investigation into the mechanisms involved.

The biomass (Figure 2-2) and lipid (Figure 2-3) yields from experiment 2 as a function of pH and temperature also show that there are some pH-media-temperature interactions. The most apparent pH-media-temperature interaction occurred at 10 °C (Figure 2-2) where there was decreased growth in PDB and DYB and no noticeable growth in the CMB medium. This specific interaction may be due to the nutritional

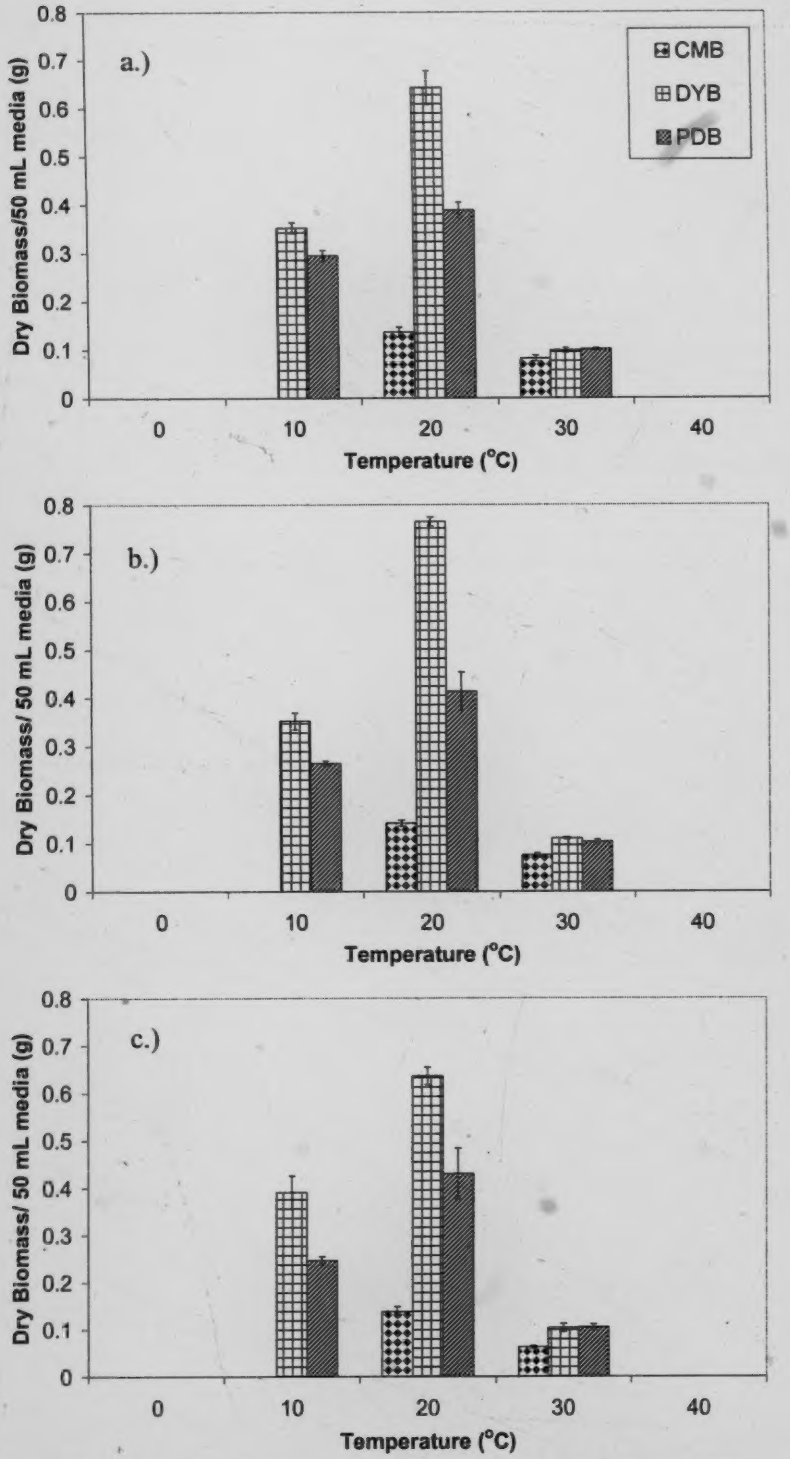


Figure 2-2 : Dry biomass yields per 50 ml of media at different pH's and temperatures. (CMB = cornmeal infusion broth; DYB = dextrose yeast broth; PDB = potato-dextrose broth). (a) pH 3 (b) pH 6 (c) pH 9

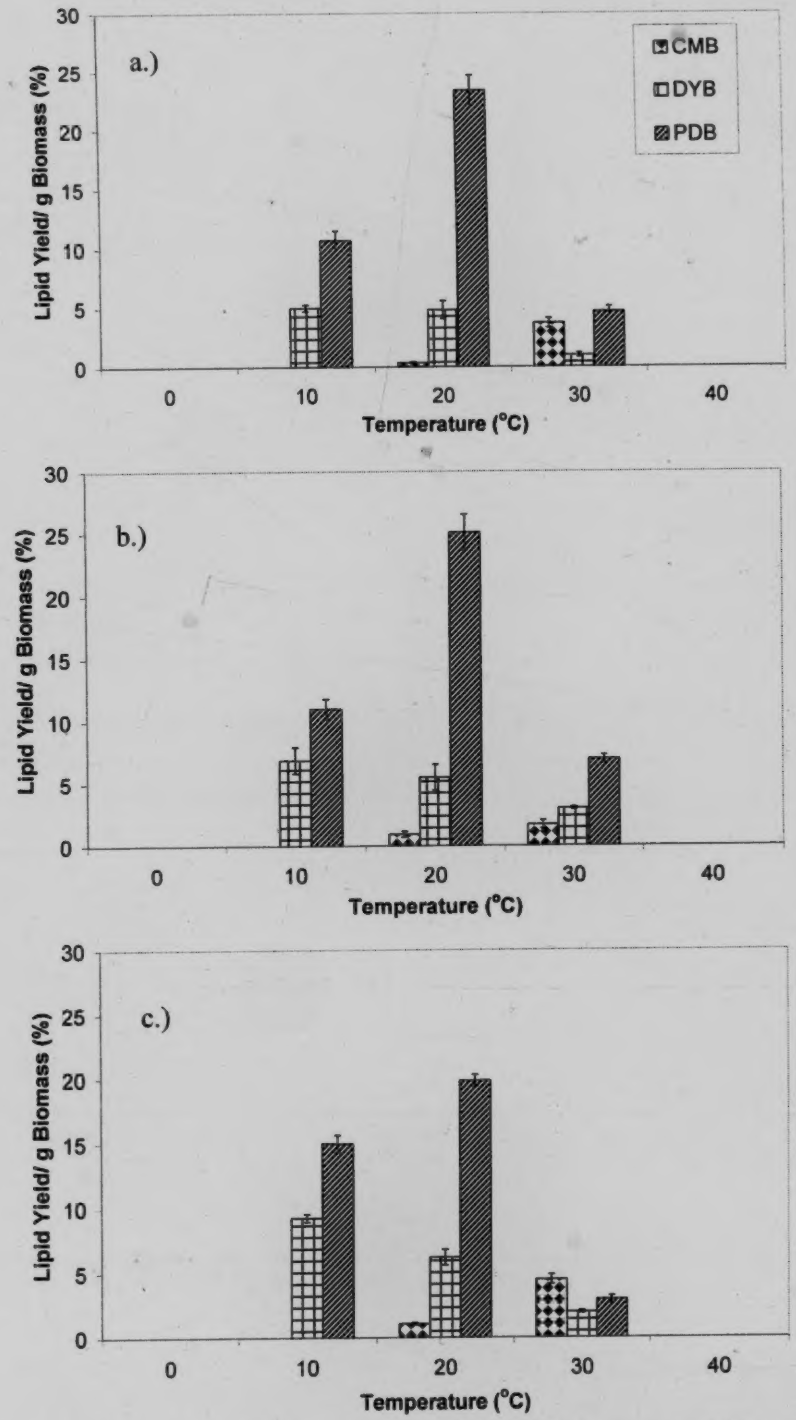


Figure 2-3: Lipid yields as a percentage of dry biomass at different pH's and temperatures. (CMB = cornmeal infusion broth; DYB = dextrose yeast broth; PDB = potato-dextrose broth). (a) pH 3 (b) pH 6 (c) pH 9

composition of the CMB medium which makes it a poor growth medium for this fungus. Since enzymes require certain substrates with which to work, it is possible that the nutritional components of this medium cannot be efficiently utilized for growth and lipid synthesis and/or accumulation by this fungal strain. This may explain the relatively low dry biomass (Figure 2-2) and lipid (Figure 2-3) yields of fungi grown in CMB when compared to the other two media used. Furthermore, in addition to the pH range, there is also a temperature range within which an enzyme can effectively work. As such, there is an optimum pH-temperature condition under which a given enzyme can efficiently utilize its substrate. It is possible that the media-pH-temperature interaction that was observed in experiment 2 can be explained by the fact that the fluctuations seen in the recorded biomass and lipid yields are a direct reflection of the effective working range of the enzymes of this fungal species. Therefore, the lack of growth in the CMB growth media at 10 °C may be due to the fact that the fungal enzymes can utilize the nutritional components to some degree at the higher temperatures but is unable to do so at lower temperatures.

The biomass results from experiment 2 (Figure 2-2) demonstrate a consistent maximum yield at 20 °C for all three media across all of the pH's tested, although at this temperature slightly higher yields in CMB were recorded at pHs 6.0 and 9.0, the highest yield in DYB was recorded at pH 6.0 (while the yields in this medium were the same at pHs 3.0 and 9.0) and there were slightly higher yields in PDB at pHs 6.0 and 9.0. The growth condition that yielded the highest biomass overall was DYB growth medium at pH 6.0 and at 20 °C (0.7650 g). At 10 °C, the biomass yields obtained in each medium remained constant across the pH range. For example, in the DYB growth medium, growth at pH 3.0 (0.35 ± 0.01g) were similar to those recorded at pH 6.0 (0.35 ± 0.02 g) and pH 9.0 (0.39 ± 0.03 g). Note that at 10 °C, fungi in the CMB medium demonstrated negligible growth. The yields for biomass at 30 °C were the lowest recorded in the PDB and DYB media, with very little variation in pH. Although the growth in CMB at 30 °C remained relatively consistent across the pH range, growth in this medium at 30 °C was higher than growth demonstrated at 10 °C, but less than that at 20 °C. The trends observed for all the media at the various temperatures and pH's suggest that there are media-temperature interactions occurring that affect the growth of this *M. ramanniana* var. *ramanianna* strain. However, clearly the optimum pH and temperature would be pH 6.0 and 20 °C, in all media.

The lipid yields for experiment 2 are shown in Figure 2-3 and are presented as percent lipid yield per biomass (w/w). There were clear temperature-pH-media interrelationships in terms of lipid yield. For example, at 10 °C, lipid yield within the PDB growth medium was highest at pH 9.0, and relatively constant between pH 3.0 and 6.0. At 20 °C and 30 °C, lipid yield was highest at pH 6.0 and lowest at pH 9.0. Overall, the lipid yields at 20 °C were the highest recorded across the pH range, and those at 30 °C the lowest. Within the DYB medium, the lipid yields increased with increasing pH at all temperatures except for 30 °C, where the yield increased from pH 3.0 to 6.0, but fell at pH 9.0. The highest lipid yields within the DYB medium were obtained at pH 9.0 and at 10 °C. It is difficult to determine whether any trends were present in the CMB data since there was no growth at 10 °C. However, at each pH tested, the highest lipid yield for CMB was obtained at 30 °C.

In a study by Leman and Brakoniecka-Sikorska (22), it was observed that the highest biomass and lipid yields were produced at pH 4.5 and at a temperature of 30 °C for a strain of *M. ramanniana* var. *angulispora*. However, Hansson and Dostalek (19) found that the optimal growth temperature for two *M. ramanniana* strains was 25 °C while 20 °C favoured GLA production. The results from these studies, coupled with those obtained from this current experiment shows that, even within the species *M. ramanniana*, there are considerable differences in how various growth factors and their interactions can affect the biomass and lipid yields of a given fungal strain. Clearly, experiments such as the ones being reported in this publication are important for the establishment of optimum growth conditions for all *Mortierella* strains.

Based on the results from our current study, the third experiment in this series was performed at room temperature (22.4 ± 0.3 °C) and without adjusting the final pH of the media. The reasoning was that the ultimate goal of these experiments is to optimize the growth conditions of this *M. rammaniana* var. *ramanniana* strain for industrial production of GLA. As such, it is important to keep costs down and limit the potential for contamination of the GLA source (i.e., the fungal cultures). Although the highest percent lipid yield was observed at 10 °C for DYB, the highest biomass yield was obtained at 20 °C and, therefore, the highest absolute lipid yield for DYB was recorded at 20 °C. Both the highest lipid and biomass yield was observed at 20 °C for PDB, therefore, its highest absolute lipid yield was also at 20 °C. Room temperature (22.4 ± 0.3 °C) is close enough to 20 °C to be a viable temperature for further optimization. On an industrial scale, this option could potentially keep down the cost of production since extensive temperature control measures would not have to be put in place to bring the cultures to the necessary growth temperature. However, some cooling devices may need to be implemented to counteract the heating effects of mechanical devices used during culture growth and the heat of metabolism released during growth of the fungus itself. The results for CMB were ignored, since CMB proved to be a poor growth medium for this *M. ramanniana* strain. Also, since this particular *M. ramanniana* strain is relatively pH insensitive, it was decided that the pH's of the media used in experiment 3 would not be adjusted, however, their final pH's after autoclaving were recorded (Table 2-1). This option also allows for cost cutting measures on an industrial scale since extra resources would not have to be put into adjusting the pH of the growth media. This would also eliminate a step where possible contamination of the fungal cultures could occur.

Experiment 3 tested various carbon sources at different concentrations to determine what effect these two variables had on dry biomass, lipid yield, fatty acid profile and GLA content. The yield of dry biomass per 50 mL of media is shown in Figure 2-4. Beet pulp was the poorest carbon source and only yielded detectable biomass at concentrations of 1% and 2% (0.0989 g and 0.1057 g, respectively). Sodium acetate was the second poorest carbon source and only yielded biomass at carbon concentrations 1%, 2% and 5% while the simple sugars (dextrose, sucrose, fructose) yielded the best results, overall. These results are similar to those obtained by Sajbidor *et al.* (24) who found that sodium acetate was the poorest carbon source for the growth of *M. ramanniana* 1022 when compared to the other carbon sources they tested (i.e., simple sugars, soluble starch).

The dry biomass results from experiment 3 (Figure 2-4) showed a general trend of increasing biomass with increasing carbon concentration for most of the growth media.

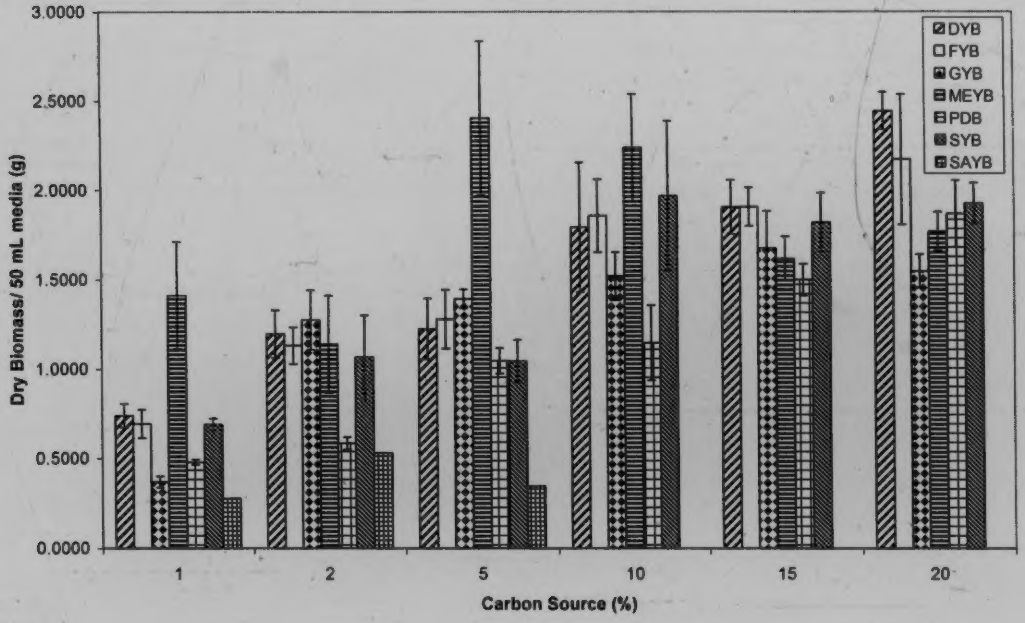


Figure 2-4: Dry biomasses per 50 ml of media using different carbon sources at room temperature. (DYB = dextrose yeast broth; FYB = fructose yeast broth; GYB = glycerol yeast broth; MEYB = malt extract yeast broth; PDB = potato-dextrose broth; SYB = sucrose yeast broth)

Notable exceptions were: GYB, in which biomass yield increased with increasing concentration, but peaked at 15% carbon source; MEYB, which demonstrated a irregular yield of biomass with the highest yield being at 5% carbon source; and SYB, which demonstrated a biomass yield increase with increasing carbon percent up to 10%, and then demonstrated a decrease with increasing carbon concentration. The DYB and FYB media yielded similar biomass results at all carbon concentrations. These results are similar to those reported by Hansson and Dostalek (19) in which the observed biomasses for fungi grown in media containing fructose as the carbon source were slightly higher than those containing dextrose (12.0 g/L and 11.2 g/L, respectively). Both the fructose- and dextrose-containing media yielded much higher biomass results than the sucrose-containing media (8.7 g/L). Our results are in agreement with the results reported by these researchers.

Figure 2-5 shows the lipid yields per g biomass as a function of growth medium and carbon source percent. There are no lipid data shown for SAYB or the beet pulp-containing media since the lipid yields were undetectable. The trends for biomass yield were not mirrored by lipid yields. The maximum lipid yield was obtained at a carbon concentration of 5% for DYB, PDB, and SYB, 10% for FYB, and 2% for MEYB. However, the calculated error values for the lipid yields in the FYB medium reported for the 5% and 10% carbon concentrations overlap. Therefore, it is possible that lipid accumulation for the FYB growth medium could also be optimized at the 5% concentration. Similarly, the results indicate that the optimum carbon concentration for lipid accumulation using MEYB as a medium was 2%. However, the calculated error

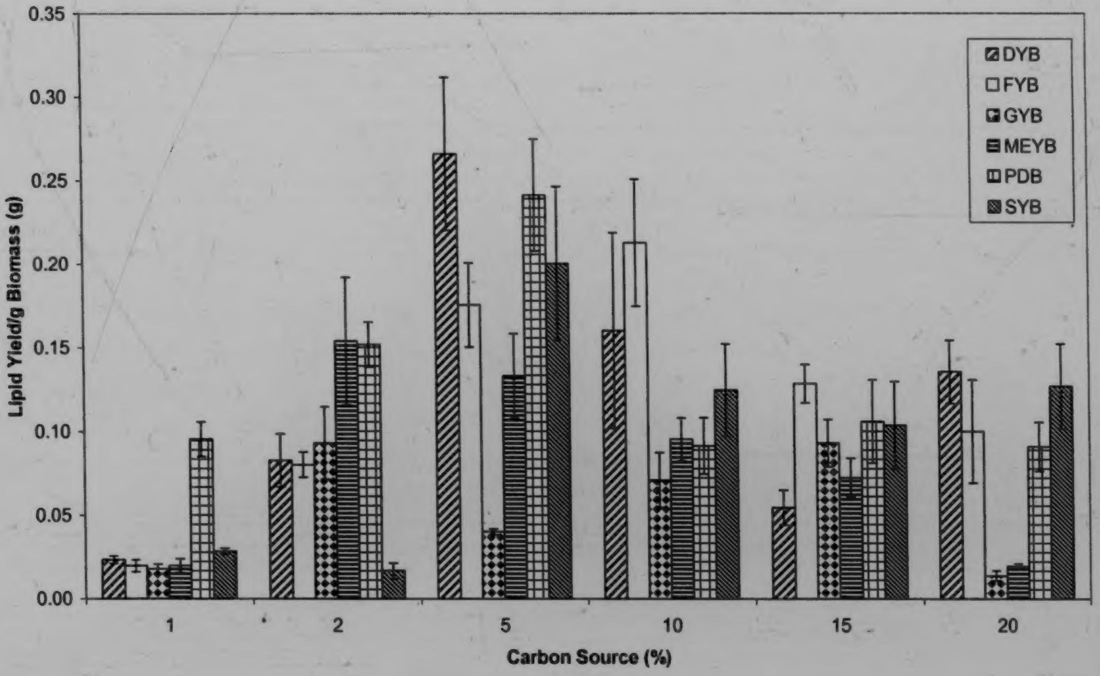
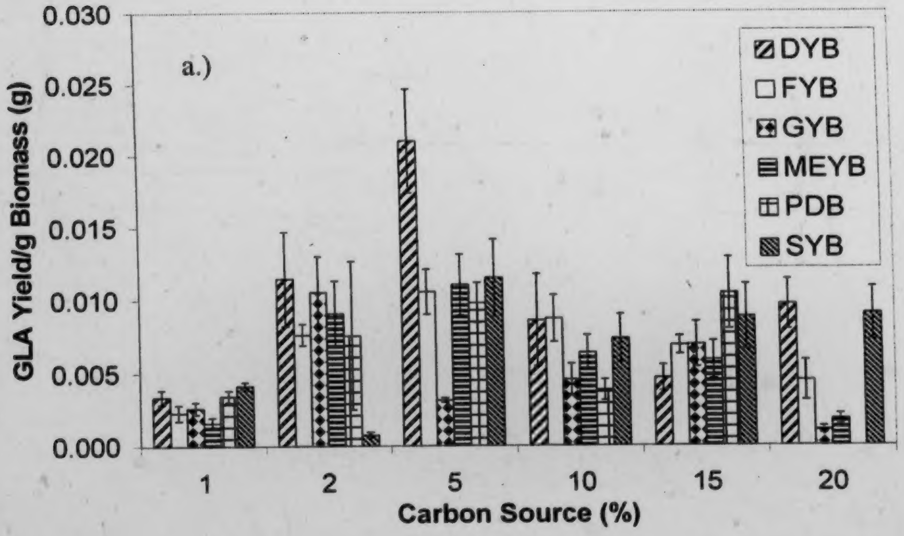


Figure 2-5: Lipid yields per g biomass using different carbon sources at room temperature. (DYB = dextrose yeast broth; FYB = fructose yeast broth; GYB = glycerol yeast broth; MEYB = malt extract yeast broth; PDB = potato-dextrose broth; SYB = sucrose-yeast broth)

values for the 2% and 5% concentrations overlap in such a way that it may be possible to optimize lipid yield in MEYB at 5%.

The lipid data that was recorded for the GYB growth media was more erratic than the results obtained for the other media and there is no clear carbon concentration for maximum lipid yield (highest lipid yields were recorded at 2% and 15%). The only major difference between GYB and the other media is that the other media contain simple sugars as part of their composition. The simple sugar media (DYB, FYB, SYB) have obvious sources of these sugars but both PDB and MEYB also contain these carbon sources. PDB contains dextrose in addition to its potato starch and MEYB contains maltose in addition to other simple sugars. The glycerol that serves as the carbon source in GYB is a sugar alcohol. Since *Mortierella* spp. are fungi that are known to readily utilize simple sugars (31), it may be that they are not as effective at utilizing a sugar alcohol. In order to determine whether this hypothesis is true, further studies would have to be performed to compare the lipid yields obtained using various simple sugar sources and a number of sugar alcohol sources at varying C/N ratios.

A complete fatty acid profile was obtained for all the media tested in experiment 3 and the results are shown in Figures 2-6 and 2-7. The error bars in the graphs represent the absolute error calculated for each value (n = 4). The target fatty acid for these experiments was GLA and the absolute values for this fatty acid are shown in Figure 2-6a (per gram biomass). Dextrose yielded the highest absolute GLA value at 5% carbon source (Figure 2-6a). It is also interesting to note that at this carbon concentration, DYB also gave the highest LA yield (Figure 2-6b), another *omega*-6 fatty acid that is the immediate precursor to GLA.



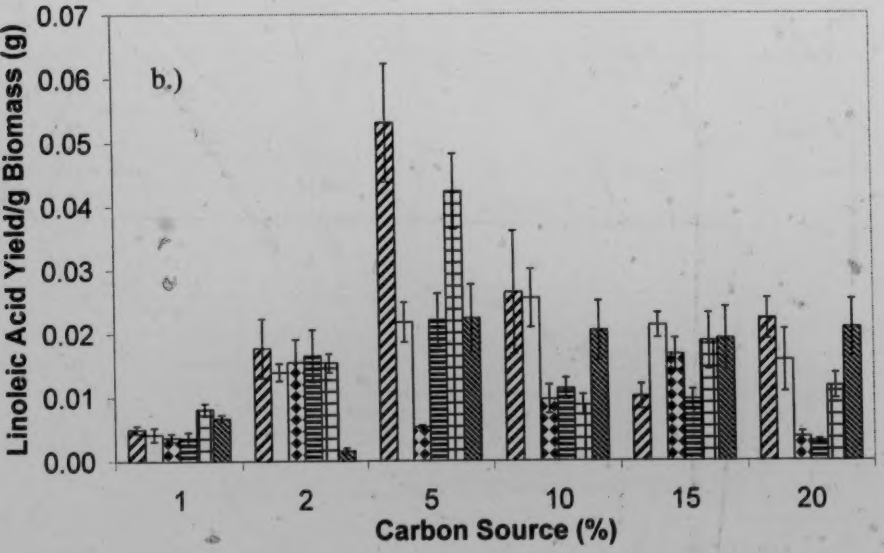


Figure 2-6: Absolute yields of unsaturated fatty acids per g biomass using different carbon sources at room temperature. (DYB = dextrose yeast broth; FYB = fructose yeast broth; GYB = glycerol yeast broth; MYB = malt extract yeast broth; PDB = potato-dextrose broth; SYB = sucrose-yeast broth) (a.) *Gamma*-linolenic acid (b.) Linoleic acid

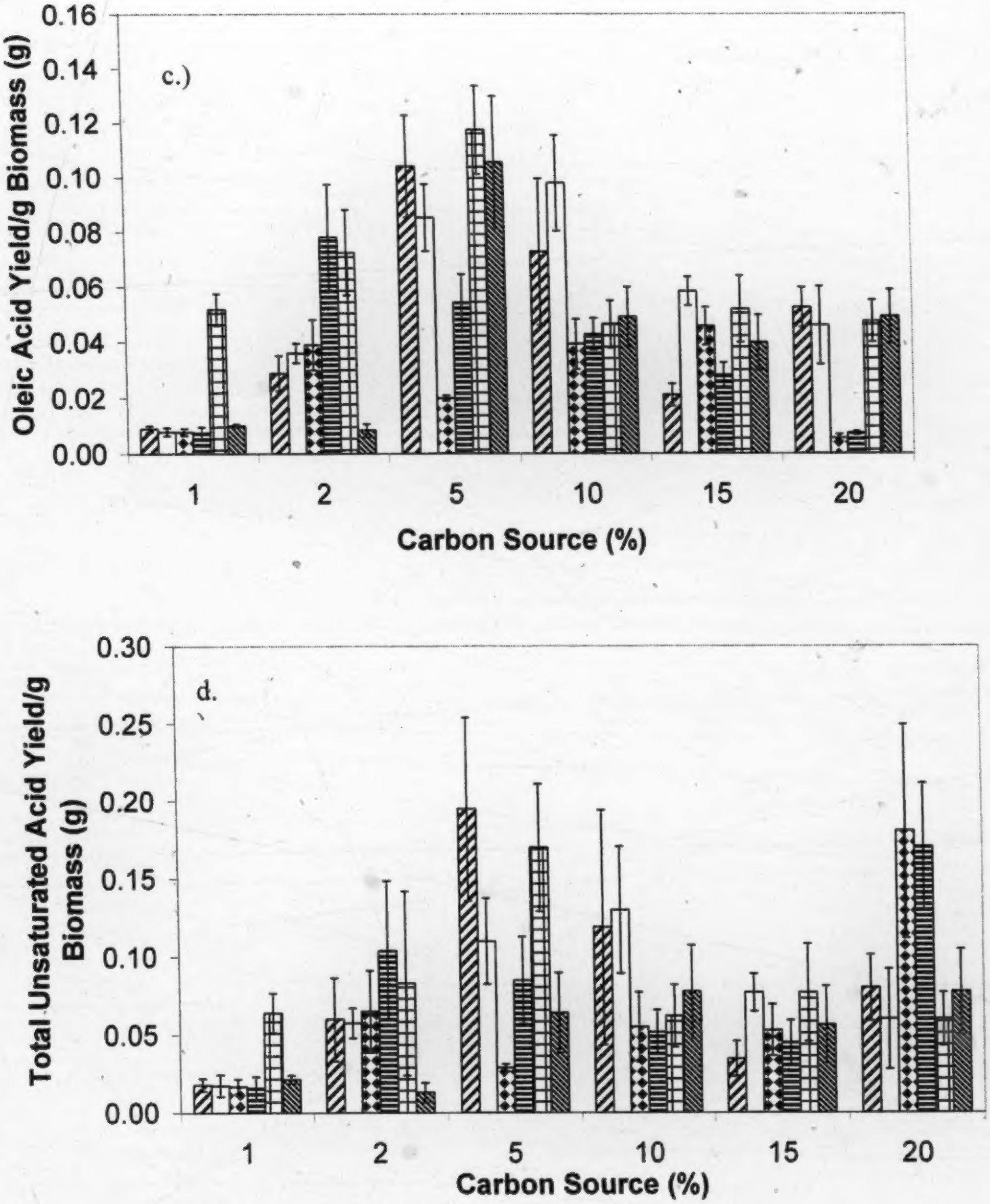
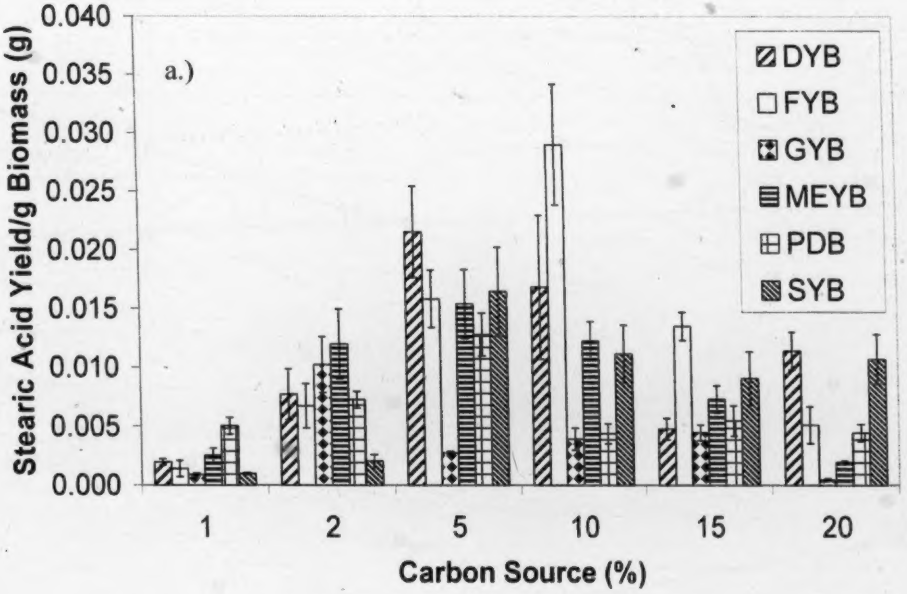


Figure 2-6: Absolute yields of unsaturated fatty acids per g biomass using different carbon sources at room temperature. (DYB = dextrose yeast broth; FYB = fructose yeast broth; GYB = glycerol yeast broth; MYB = malt extract yeast broth; PDB = potato-dextrose broth; SYB = sucrose-yeast broth) (c.) Oleic acid (d.) Total unsaturated fatty acids



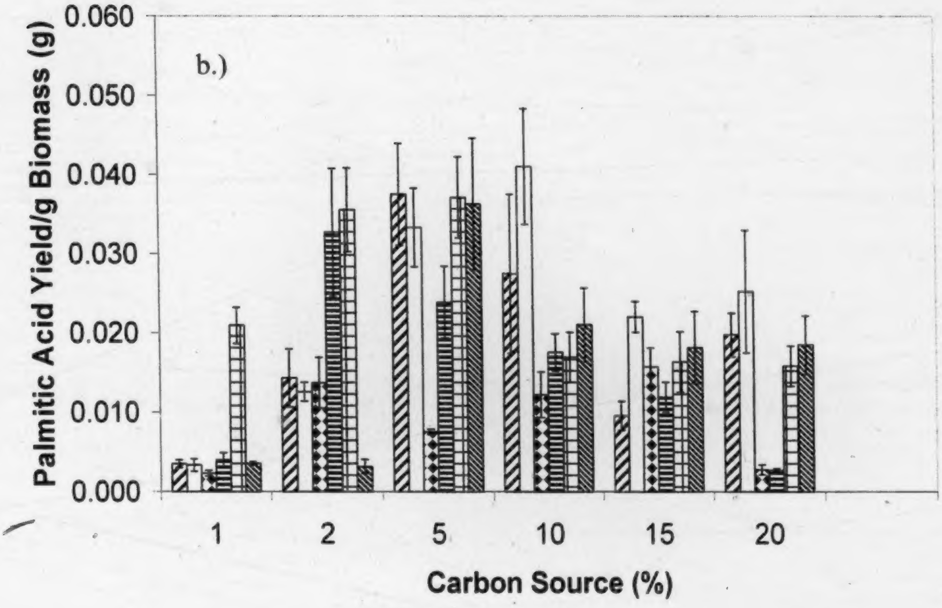
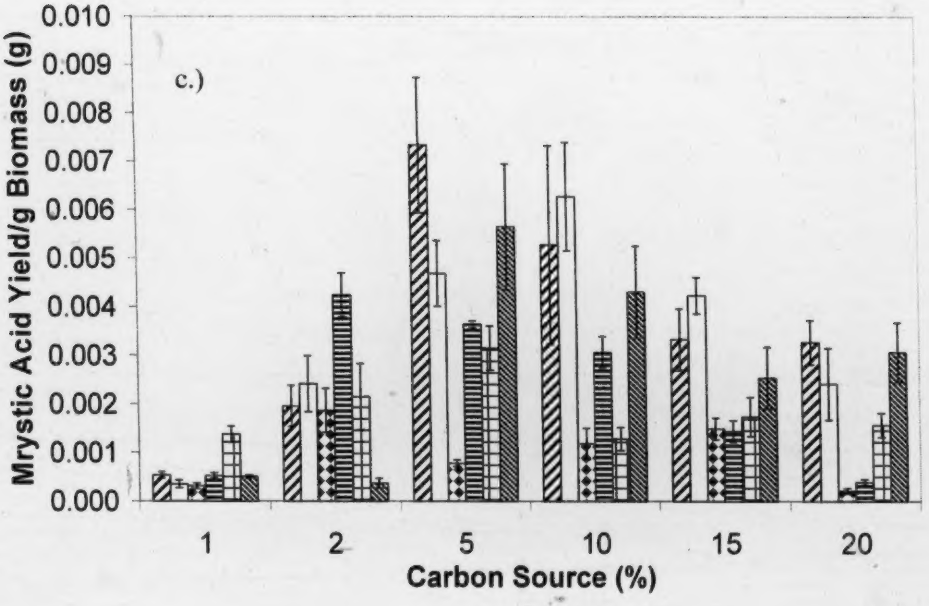


Figure 2-7: Absolute yields of saturated fatty acids per g biomass using different carbon sources at room temperature. (DYB = dextrose yeast broth; FYB = fructose yeast broth; GYB = glycerol yeast broth; MEYB = malt extract yeast broth; PDB = potato-dextrose broth; SYB = sucrose-yeast broth) (a.) Stearic acid (b.) Palmitic acid



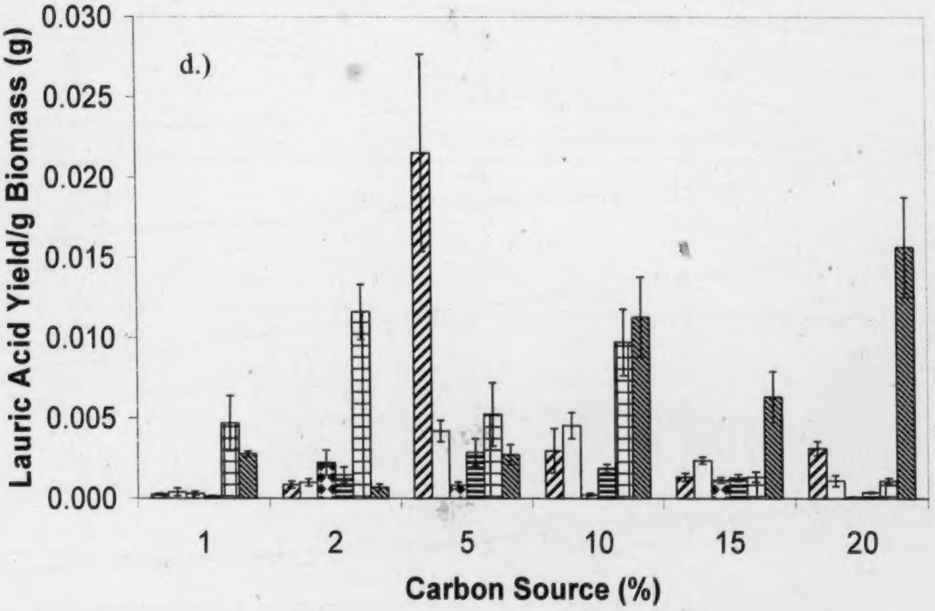


Figure 2-7: Absolute yields of saturated fatty acids per g biomass using different carbon sources at room temperature. (DYB = dextrose yeast broth; FYB = fructose yeast broth; GYB = glycerol yeast broth; MEYB = malt extract yeast broth; PDB = potato-dextrose broth; SYB = sucrose-yeast broth) (c.) Mrystic acid (d.) Lauric acid

The general trends of each of the fatty acids within a given medium followed similar patterns to those of the lipid profiles obtained for that medium (Figures 2-6 and 2-7). It is interesting to note how readily the fatty acid composition of *Mortierella* fungi can be manipulated by simply varying the concentration and source of the carbon in the fungal growth media. The total unsaturated fatty acid yield ranged from 0.0397 g/g biomass to 0.1952 g/g biomass (Figure 2-6d). Though all of the fatty acids (both saturated and unsaturated) showed large variations in their yields as a function of both carbon source and carbon concentration, the most dramatic differences can be found in the values obtained for lauric acid (Figure 2-7d). The maximum yield of this fatty acid was observed with DYB at a 5% carbon concentration (0.0215 ± 0.0050 g/g biomass) and its minimum recorded value was 0.0001 g/g biomass.

The maximum lipid yield was also achieved with 5% DYB (0.2763 g/g biomass) as was the maximum unsaturated fatty acid yield (0.1952 g/g biomass) and GLA yields (0.0226 g/g biomass). This indicates that 70.6 % of the fungal lipid is in the form of unsaturated fatty acids. Of this, GLA makes up 11.6% of the unsaturated fatty acid yield (8.3% of the total fatty acid yield) which amounts to 8.2% of the total lipid yield. Evening primrose oil typically contains between 3.00% - 15.00% of its total fatty acid yield as GLA (Table 1-1). This suggests that this fungal strain could be an at least comparable industrial source of GLA to evening primrose oil.

Table 2-2 shows some GLA yields (as a percent of the total fatty acid yield) of a number of other fungal sources which were obtained using various growth conditions. The current set of experiments reports GLA yields that are in the range of those previously reported for various *M. ramanniana* strains. However, the GLA contents

Table 2-2: Previously reported GLA contents (% total fatty acid yield) for various fungal sources.

Fungal Species	Reference	GLA content (% total fatty acid)
M. ramanniana	Amano *et al.* (7)	13.2 – 31.4%
	Burranova *et al.* (8)	9.5 – 16.3%
	Hansson and Dostalek (14)	4.1 – 4.4%
	Kavadia *et al.* (18)	9.7%
	Leman and Brakoniecka (24)	3.0 – 6.0%
	Nakahara *et al.* (26)	4.8 – 21.5%
	Stredanska and Sajbidor (34)	7.6%
M. isabellina	Amano *et al.* (7)	6.9 – 22.9%
	Buranova *et al.* (8)	7.6 – 11.6%
	Hansson and Dostalek (14)	2.7%
	Kavadia *et al.* (18)	4.3%
	Kendrick and Ratledge (21)	0.5 – 3.9%
	Nakahara *et al.* (26)	3.5 – 21.5%
	Stredanska and Sajbidor (34)	20.2%
M. vinacea	Amano *et al.* (7)	22.0%
	Buranova *et al.* (8)	6.6%
	Hansson and Dostalek (14)	3.8%
M. circinelloides	Funtikova and Mysyakina (13)	3.0 – 7.3%
	Kendrick and Ratledge (21)	1.1 – 8.3%
	Stredanska and Sajbidor (34)	28.6%
M. racemosus	Funtikova and Mysyakina (13)	2.6 – 8.0%
	Stredanska and Sajbidor (34)	11.0%
Rh. oryzae	Funtikova and Mysyakina (13)	1.6 – 6.7%
	Nakahara *et al.* (34)	5.6 – 15.3%

obtained during this project through manipulation of pH, temperature, and carbon source are still lower than the highest values obtained for *M. ramanniana* strains as well as those values obtained from other fungal species (Table 2-2). Even so, the results are still promising in light of the fact that previous studies (19 – 23) have shown that there are a number of other variables that can be manipulated to potentially further maximize the GLA yield of this organism. These include nitrogen source, addition of metal ions to the growth media, and supplementation of the growth media with various oils.

Although it is generally true that oleaginous fungi accumulate lipids when their nitrogen source has become limiting (32), there also appears to be an optimum carbon concentration at which this accumulation is maximized. In this study, it appears that these optimizations occurred at either the 5% or 10% carbon concentrations with a 1% nitrogen concentration, depending on the media used. The results of this experiment indicate that the optimum conditions for GLA production per g biomass was a temperature of 22.4 ± 0.3 °C, at the unadjusted pH of the media after autoclaving, and the best carbon source was dextrose at a carbon concentration of 5%. However, further research needs to be done to determine what effects nitrogen source, metal ion addition, and supplementation of the media with oils have on GLA production of this fungal strain.

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Chapter 3

Maximizing the Production of *Gamma*-Linolenic Acid in *Mortierella ramanniana* var. *ramanniana* as a Function of Nitrogen, Metal Ion Supplementation and Oil Supplementation

Introduction:

It is generally accepted that *omega*-3 and *omega*-6 fatty acids are vital for good health (1). As these essential fatty acids (EFAs) are essential to human health, but are not synthesized by the human body, a healthy diet needs to include these fundamental components. As mentioned in previous chapters, the introduction of foods enriched with EFAs requires an increase in the industrial production of these fatty acids. Since traditional sources of EFAs used by industry offer relatively low yields at relatively high costs (2, 3), some researchers have been attempting to find alternate, relatively inexpensive sources that produce higher yields.

The plausibility of using *Mortierella* spp. as an alternative for EFA production has been validated by the fact that oil rich in arachidonic acid (ARA) is being been extracted from *Mortierella alpina* and utilized in the production of some infant formulas (e.g., Martek Biosciences Corporation). Currently, the major source of *gamma*-linolenic acid (GLA) is evening primrose oil (2, 3) however *M. ramanniana* var. *ramanniana* offers one possible alternate source for industrial GLA production.

In the experiments performed in Chapter 2, pH, temperature, and carbon source were systematically manipulated using a locally isolated strain of *Mortierella ramanniana* var. *ramanniana* (4). It was found that the optimum conditions for GLA production (per g biomass) was a temperature of 22.4 ± 0.3 °C, at the unadjusted pH (ranging from pH 4.54 to pH 6.70) of the media after autoclaving, and the best carbon source was dextrose at a concentration of 5%. The experiments performed in Chapter 3 focused on the systematic manipulation of nitrogen source, metal ion concentration, and supplementation of the growth medium with various oils using the same fungal isolate.

Experimental Procedures:

The same strain of *Mortierella ramanniana* var. *ramanniana* used in Chapter 2 was used for this second set of experiments. As before, stock cultures were maintained in potato-dextrose agar (PDA) (Difco) and plugs were transferred to new media every ten days. Experiment 1 was performed to determine the optimum nitrogen sources and concentrations for growth, lipid yield, and absolute GLA content. The results from the experiments performed in Chapter 2 (4) established that this organism is relatively pH insensitive and can accumulate a relatively high GLA content (per g biomass) using 5% dextrose as a carbon source at room temperature (22.4 ± 0.3 °C) when 1% (w/v) yeast extract was used as the sole carbon source. The nitrogen sources tested for the current experiment included yeast extract, lysine, casein, peptone and KNO_3 each at concentrations of 1%, 0.5%, 0.25% (w/v of effective nitrogen content). Media containing 0% nitrogen source were used as controls. Five percent dextrose (w/v) was used as the carbon source for all media. The resulting media were each transferred to 250 ml Erlenmeyer flasks (100 ± 2 ml/flask) and then autoclaved for 25 minutes at 121 °C. The media were inoculated by one plug each of stock culture, covered, and left to grow in an orbital shaker set to 100 rpm at room temperature (22.4 ± 0.3 °C). After ten days of growth, the mycelia were filtered and dried as in Chapter 2 and the resulting dry biomasses were recorded for each growth condition (to 0.0001 g). Then the lipid and fatty acid yields were also determined as in Chapter 2. The highest GLA yields (per g biomass) from experiment 1 were obtained from fungi grown in the DYB growth medium containing 5% dextrose and 1% yeast extract. Therefore, this was the medium used for both the metal ion and supplementation experiments.

Experiment 2 was performed in order to determine the effect that metal ion concentration had on growth, lipid yield, and GLA content for this *M. ramanniana* strain. The metal ions that were used in this experiment were magnesium (Mg^{2+}), manganese (Mn^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), iron (Fe^{2+}), and calcium (Ca^{2+}). The first four ions were added to the media in the form of sulfates while the last two ions were in the form of chloride compounds (i.e., iron II chloride tetrahydrate and calcium chloride, respectively). Each metal ion was added to DYB medium composed of 5% dextrose and 1% yeast extract (w/v) in concentrations of 5 mg/L, 50 mg/L, and 500 mg/L (concentrations refer to the effective metal ion content). The cultures were allowed to grow for 10 days, filtered, dried and their biomasses, lipid yields, and fatty acid profiles recorded as in experiment 1.

Experiment 3 was performed to determine the effect that supplementation of the growth medium with various oils had on growth, lipid yield, and absolute GLA content. The same composition of DYB media that were used in the previous experiment were again used for this experiment and were prepared as outlined in Experiment 1. The oils tested in this experiment included safflower, sunflower, canola, sesame, olive and flax oils. The media was supplemented with 2% (w/v) of oil (20, 22). As before, the cultures were allowed to grow for 10 days before being filtered, dried and having their biomasses, lipid yields, and fatty acid profiles recorded. All experimental samples for the above three experiments were replicated in quadruplicate.

Results and Discussion:

The results from Chapter 2 indicated that this particular *Mortierella ramanniana* var. *ramanniana* isolate is relatively pH insensitive between the pH range of 3 and 10, regardless of the media used (4). The results of this previous experiment also indicated that the optimum conditions for growth, with regards to the GLA content, was at room temperature (22.4 ± 0.3 °C) and the best carbon source out of the ones tested was dextrose at a concentration of 5% when yeast extract was used at a constant concentration of 1%. Therefore, based on these results, the carbon source used for all experiments in this current study was dextrose at a concentration of 5%.

A number of researchers have tested the effect that nitrogen source has on the biomass and lipid yields of various *Mortierella* fungi (5 - 10). In those experiments that tested yeast extract as a potential nitrogen source, it was found that it was the best nitrogen source for maximizing biomass, lipid, and target fatty acid (ARA and/or EPA) yield (8 - 10). In this literature, dextrose was also used as a carbon source and results indicated that the optimum percentage of yeast extract was 1% when the dextrose concentration was held at either 2% or 4%. However, 1% was the lowest nitrogen concentration tested in these experiments.

In this current study, similarly, it was found that the highest dry biomass yields were obtained with yeast extract at each concentration tested. The highest dry biomass overall was obtained with yeast extract at a concentration of 0.5% (3.7856 ± 0.2666 g/ 100 ml media) (Figure 3-1). There was no growth obtained with *L-* lysine as a nitrogen

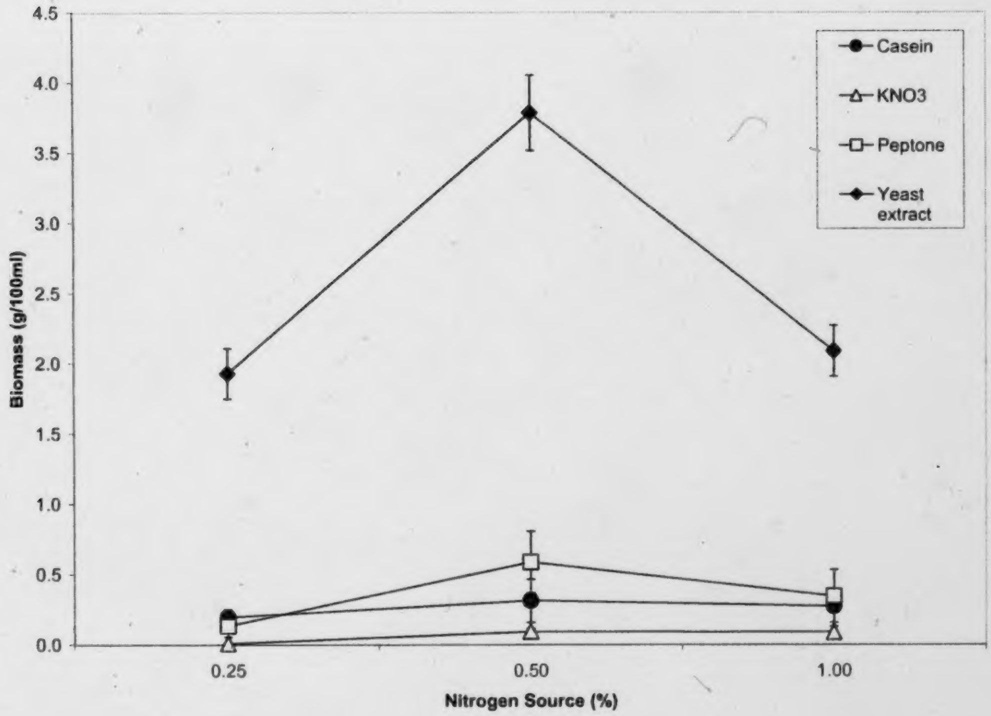

Figure 3-1: Dry biomasses per 100 ml of media obtained using different nitrogen sources..

source or with the 0% nitrogen source control. Although dry biomass yields for yeast extract were significantly higher than for the other nitrogen sources, the yields demonstrated the same pattern as a function of nitrogen concentration for *all* of the nitrogen sources tested. There was always a noticeable increase in yield for concentrations of 0.25% to 0.5% which fell at 1%, although at 1%, the yield was still higher than that at 0.25%. As would be expected by the relatively large biomass yields, the highest absolute lipid yields (per 100 ml media) were obtained with yeast extract at each concentration tested when compared to all other nitrogen sources. However, the trends observed for lipid yield per g biomass showed that, at a concentration of 0.25% nitrogen source, yeast extract yielded the lowest amount of lipid (0.0052 ± 0.0014 g/g biomass) when compared to the other nitrogen sources but had the highest lipid yield overall (0.3279 ± 0.0265 g/g biomass) at a nitrogen concentration of 1% (Figure 3-2). It is also interesting to note that yeast extract showed a direct relationship between lipid yield per g biomass and nitrogen concentration (which increased dramatically between 0.5% and 1%) while the other nitrogen sources showed an inverse relationship between these two variables (Figure 3-2).

Figure 3-3 shows the fatty acid yields per g biomass for fungi grown in yeast extract and peptone media at the varying concentrations. Fatty acid profiles could not be obtained from the KNO_3 and casein samples due to their low absolute lipid yields. The peptone-containing media showed a decreasing trend for all fatty acids with increasing nitrogen concentration (Figure 3-3a) while the yeast extract-containing media showed an increasing trend for all fatty acids with increasing nitrogen concentration (Figure 3-3b).

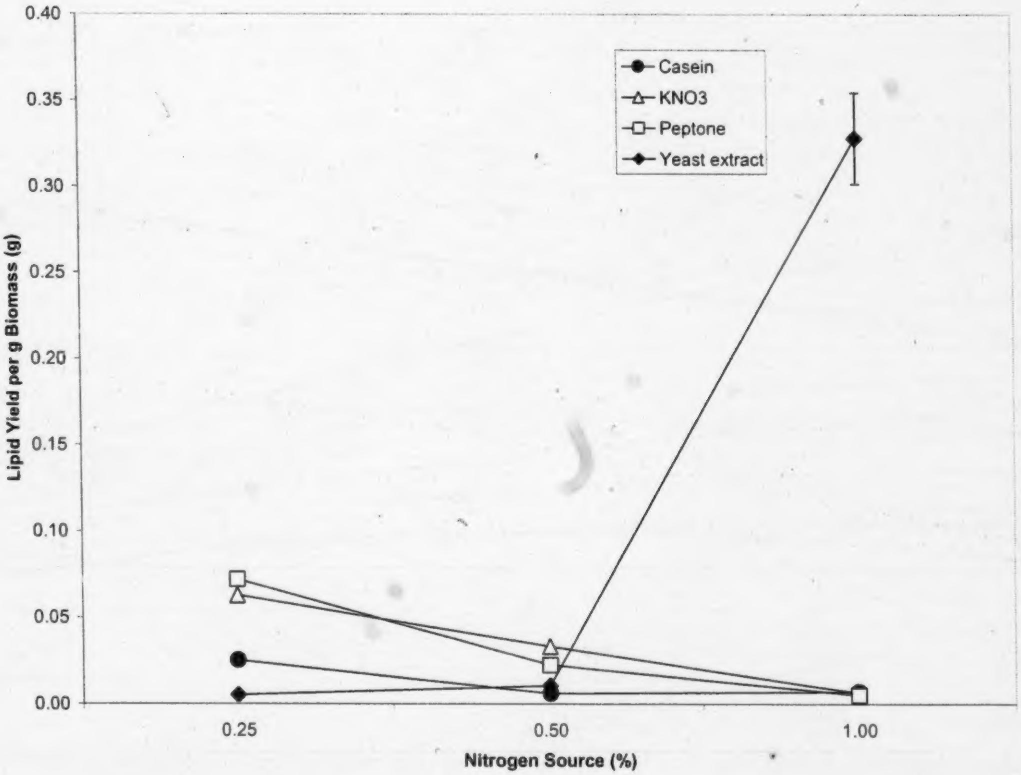

Figure 3-2 : Lipid yields per g biomass obtained for different nitrogen sources.

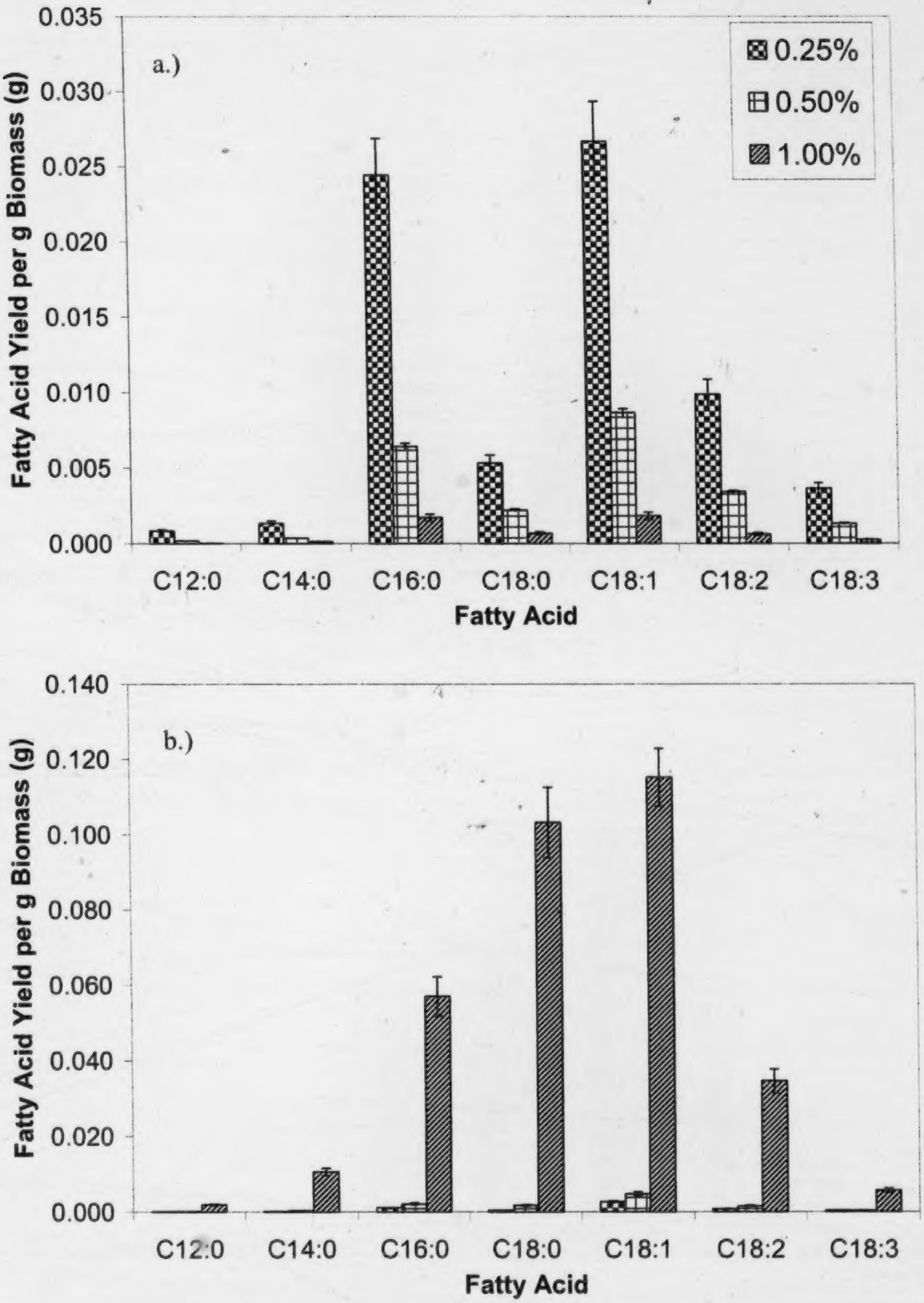

Figure 3-3: Fatty acid yields per g biomass using different nitrogen sources (a.) Peptone (b) Yeast extract

The highest GLA content per g biomass was obtained using yeast extract as a nitrogen source at a concentration of 1% (0.0226 ± 0.0052 g/g biomass). Based on these results, experiments 2 and 3 were performed using DYB media containing 5% dextrose and 1% yeast extract as the basal medium.

A previous study examined the effect of various concentrations of Ca^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+} ions on an ARA-producing *Mortierella* sp. (11). The highest lipid and ARA yields were obtained with the addition of 2 mg/L of Mn^{2+} (which was the lowest concentration tested for this metal ion). In this previous work, a general trend of decreasing lipid yield (g/L) with increasing metal ion concentration (mg/L) was observed, regardless of the metal ion tested. A similar trend was observed for ARA production.

Another study by Hansson and Dostalek (5) examined the effect that some metal ions had on the growth, lipid yield, and GLA production of *M. ramanniana*. It was found that Cu^{2+} and Zn^{2+} had positive effects on lipid accumulation and GLA production. In this study, an inverse relationship was found between biomass and metal ion concentration when Cu^{2+}, Mg^{2+}, or Zn^{2+} were added, while a direct relationship was observed between these two variables with the addition of Fe^{2+} (Figure 3-4). No obvious trend was observed in the relationship between biomass and metal ion concentration for the Ca^{2+} and Mn^{2+} ions. None of the media with added metal ions yielded biomasses higher than those obtained for the base medium itself (Figure 3-1). Figure 3-5 shows the lipid yield per g biomass as a function of metal ion concentration. None of the metal ions showed significant trends across concentration once the standard error was taken into consideration. However, an almost significant relationship was observed for Mg^{2+} which decreased with increasing metal ion concentration (Figure 3-5). The lipid yields per g

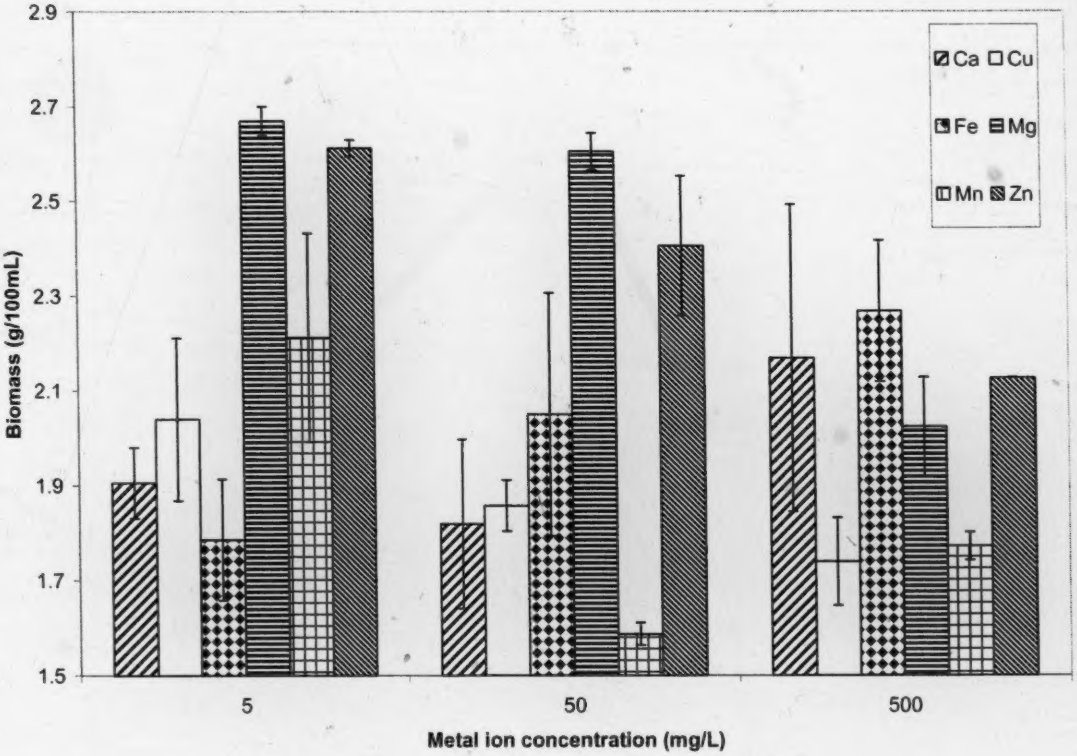

Figure 3-4: Dry biomasses per 100 ml of media obtained with the addition of different metal ions.

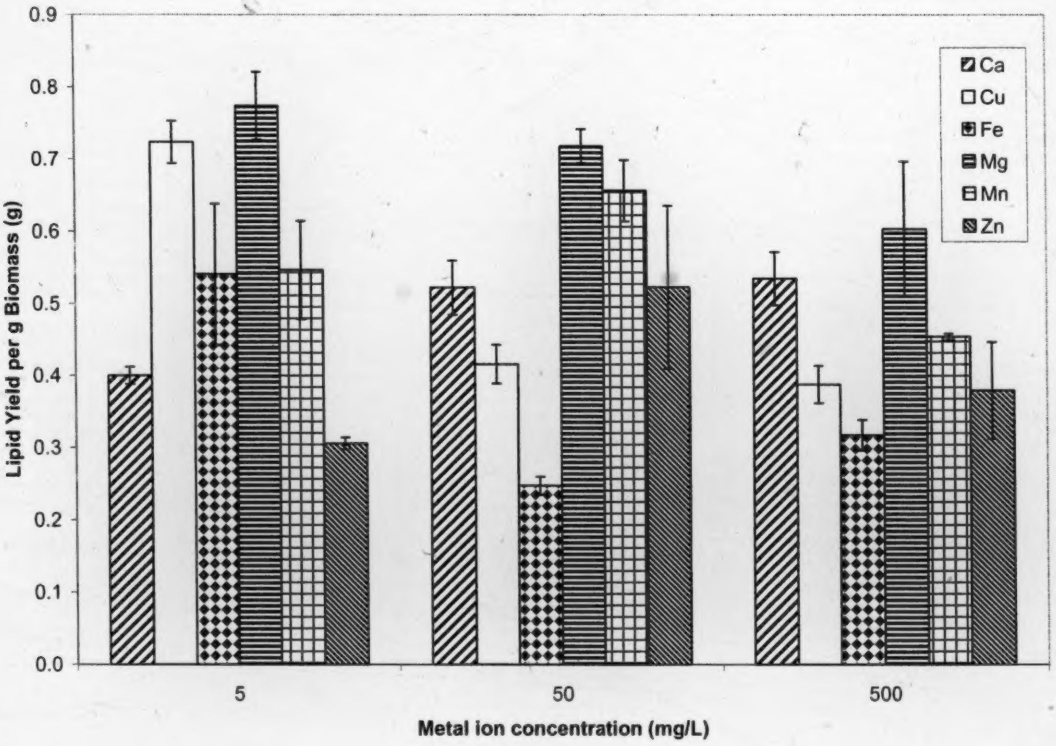

Figure 3-5: Lipid yields per g biomass obtained for different metal ions.

biomass supplemented with metal ions were higher than those obtained with the unsupplemented media (Figure 3-2) with the exception of Zn^{2+} at 5 mg/ L and Fe^{2+} at 50 mg/L and 500 mg/L. The highest GLA yield was obtained at 5 mg/L with Mn^{2+} (0.0656 ± 0.0051 g/ g biomass) (Figure 3-6c). Mn^{2+} also gave a maximum GLA yield at 500 mg/ L (0.0594 ± 0.0031 g/ g biomass) (Figure 3-6a). These yields were significantly higher than those obtained with the basal medium alone (Figure 3-3b). Contrary to the results reported by Hanson and Dostalek (13), Cu^{2+} actually had a negative affect on GLA production in the strain of *M. ramanniana* tested in this experiment. In fact, the highest GLA content obtained with the addition of Cu^{2+} was 0.0010 g/g biomass at a concentration of 50 mg/L while no detectable GLA was produced at 5 mg/L or 500 mg/L Cu^{2+} (Figure 3-6b).

It is interesting to note the variations in unsaturated fatty acid yields as a function of metal ion type and concentration (Figure 3-6a, 3-6b, and 3-6c). At concentrations of 5 mg/L and 500 mg/L, supplementation with Mg^{2+} produced the highest yield of OA (0.1912 ± 0.0147 and 0.2319 ± 0.0122 g/g biomass, respectively) whereas at 50 mg/L, the addition of Ca^{2+} produced the highest OA yield (0.1843 ± 0.0108). The highest yields of LA were produced by supplementation with Mg^{2+} at concentration of 5mg/L (0.2792 ± 0.0164 g/g biomass) and with Ca^{2+} at 50 mg/L (0.0507 ± 0.0030 g/g biomass) and 500 mg/L (0.0926 ± 0.0049 g/g biomass). Maximum yields of yields of GLA were produced by Mn^{2+} 5 mg/L and 500 mg/L and by Ca^{2+} at 50 mg/L (0.0381 ± 0.0022 g/g biomass). Clearly, not only is lipid yield per unit biomass affected by the type and concentration of metal ion added, but so is the fatty acid profile. Similar observations have been made by other researchers (5, 11).

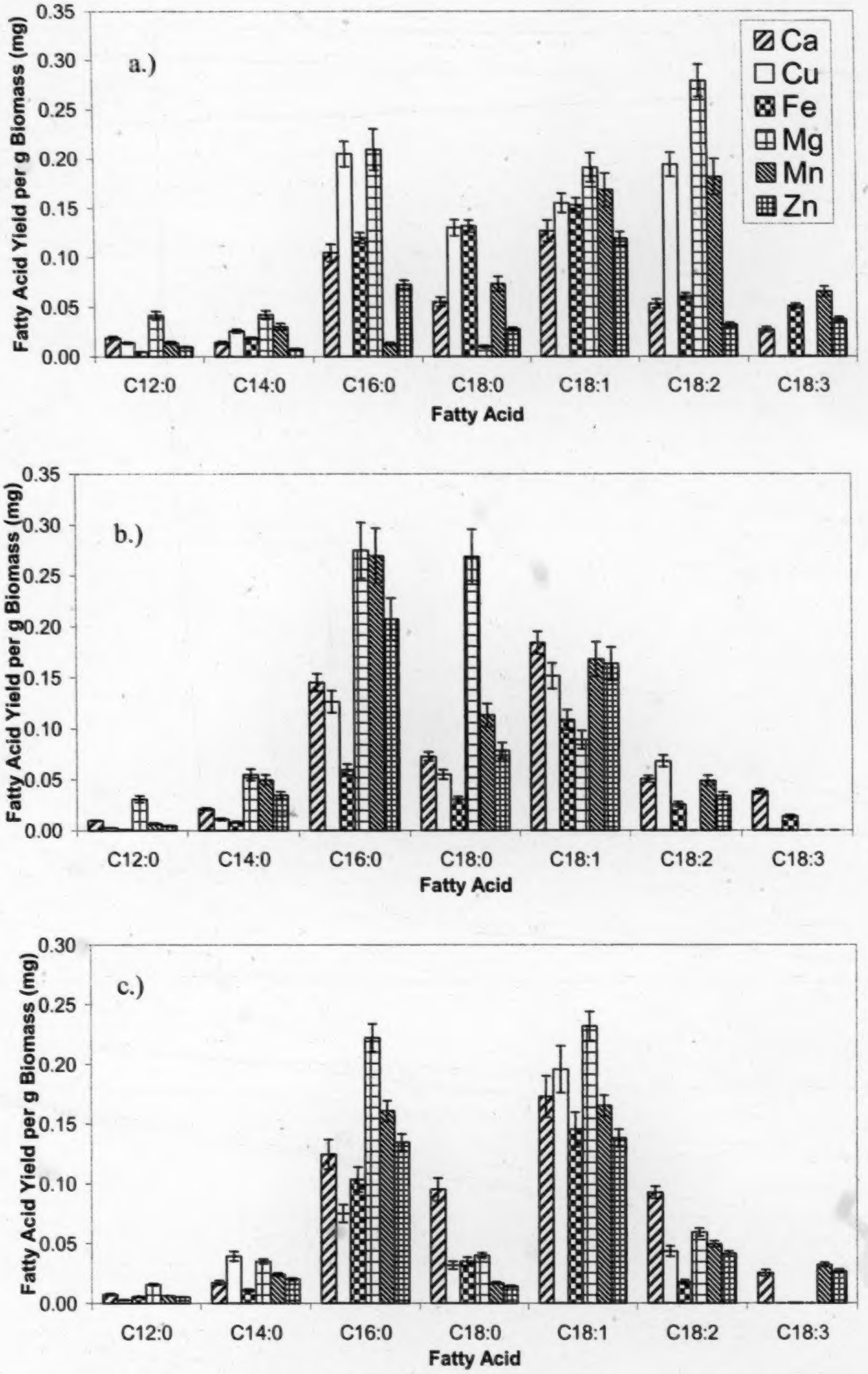

Figure 3-6: Absolute yields of fatty acids per g biomass with the addition of different metal ions (a.) 5 mg/L (b.) 50 mg/L (c.) 500 mg/L

The idea behind the supplementation of fungal growth media with various oils is that, unlike conventional carbon sources, these oils provide fatty acid precursors for long chain target fatty acids. Therefore, rather than having to synthesize these fatty acids *de nova*, the fungi can modify those provided by the oils used for media supplementation. Previous studies that have studied the affects of supplementation of fungal growth media with oils focused on *Mortierella* fungi that can produce C20 PUFAs. The main target fatty acids in these studies were arachidonic acid (ARA), dihommogamma-linolenic acid (DHGLA) and eicosapentaenoic acid (EPA). The immediate precursor for DHGLA is GLA and DHGLA itself is the immediate precursor to ARA. Research has shown that the enzyme responsible for the conversion of DHGLA to ARA (*delta*5-desaturase) is inhibited by the presence of sesame oil in the growth media (12 - 15). A number of studies have also been performed using linseed (flax) oil (16 - 18) which contains a large proportion of *alpha*-linolenic acid (ALA), a precursor of EPA. *Mortierella ramanniana* cannot synthesize C20 PUFAs but the target fatty acid in this current study was GLA. The immediate precursor to GLA is LA. This suggests that any oil containing LA could potentially enhance GLA production in this fungus.

In this experiment, the highest biomasses were obtained with the supplementation of media with canola and olive oils (2.8877 ± 0.2950 g/ 100 ml media and 2.9037 ± 0.3176 g/ 100 ml media, respectively) (Figure 3-7). However, these biomasses were lower than that recorded for the unsupplemented base medium (Figure 3-1). The lowest biomass was obtained using sunflower oil as an additive to the basal medium (2.1577 ± 0.1735 g/ 100 ml media). Figure 3-8 shows the lipid yield per unit biomass as a function of oil type. It is interesting to note that canola oil had the highest lipid yield (0.6591 ±

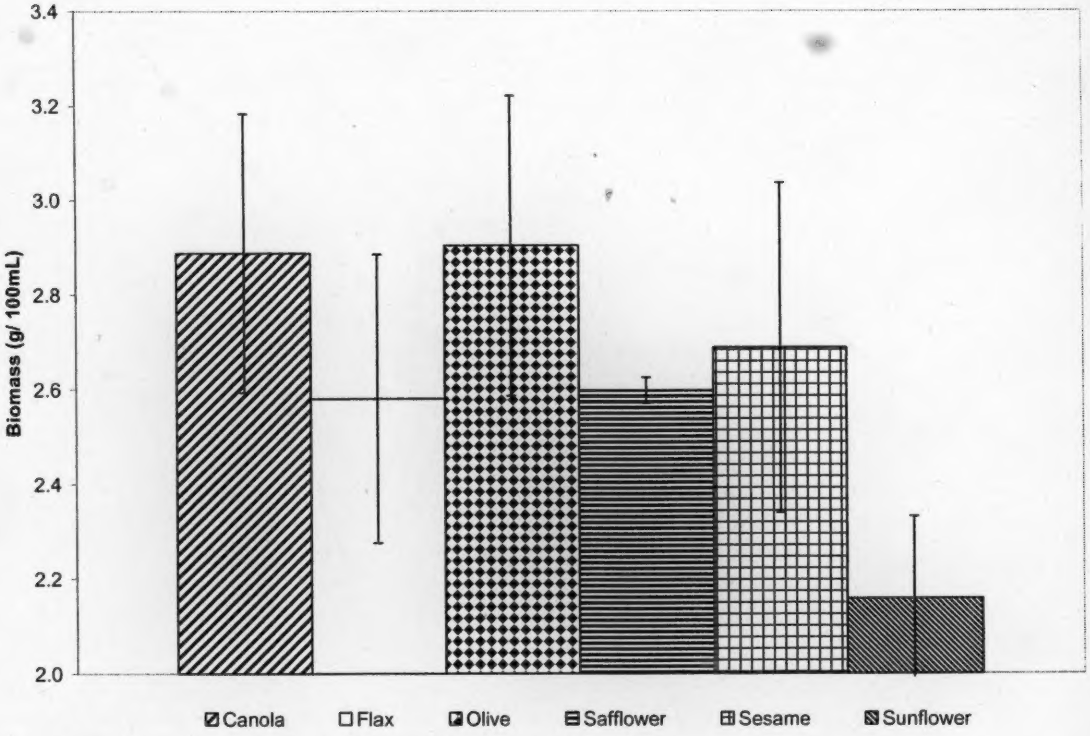

Figure 3-7: Dry biomasses per 100 ml of media supplemented with various oils

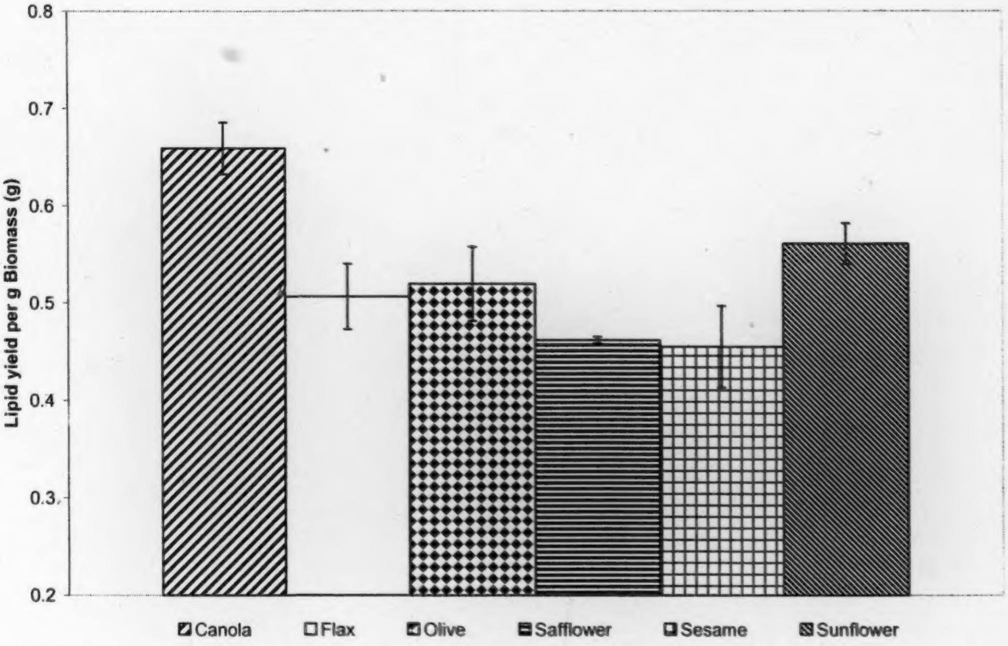

Figure 3-8: Lipid yields per g biomass for oil for supplemented media.

0.0170 g/g biomass), indicating some interactions between lipid yield/biomass ratios and oil supplementation. The fatty acid profiles obtained with media supplementation with oils are shown in Figure 3-9. Media supplemented with either sunflower or canola oils yielded a fungal oil that contained no GLA. However, the canola-containing media resulted in the highest OA yield (0.5194 ± 0.0236 g/g biomass). Media containing sesame oil resulted in fungal oil containing the highest LA yield (0.1735 ± 0.0101 g/g biomass), but it was the addition of flax oil to the media which resulted in fungal oil containing the highest GLA yield (0.0200 ± 0.0013 g/g biomass). This GLA yield per g biomass was similar to that obtained from the basal medium itself (Figure 3-3b).

The degree of enhancement that a given oil produces in GLA production needs to be weighed against the added cost that oil supplementation of the fungal growth media would add on an industrial scale. Oil supplementation would also mean that the fungal production of GLA would ultimately require the use of agricultural land. Since one of the advantages of using fungi as alternate sources of EFAs would be to minimize the need for agricultural land in EFA production, GLA production with oil supplementation must be significantly increased over and above baseline GLA production in order to justify its use in industrial GLA production.

The results from the oil supplementation experiment suggest that there would be some benefit to adding oil to the fungal growth medium for the industrial production of GLA. The results from this current study indicate that the addition of flax oil would yield similar amounts of GLA produced by this fungus when compared to using the basal medium alone. Similarly, the results from the metal ion experiment suggest that there

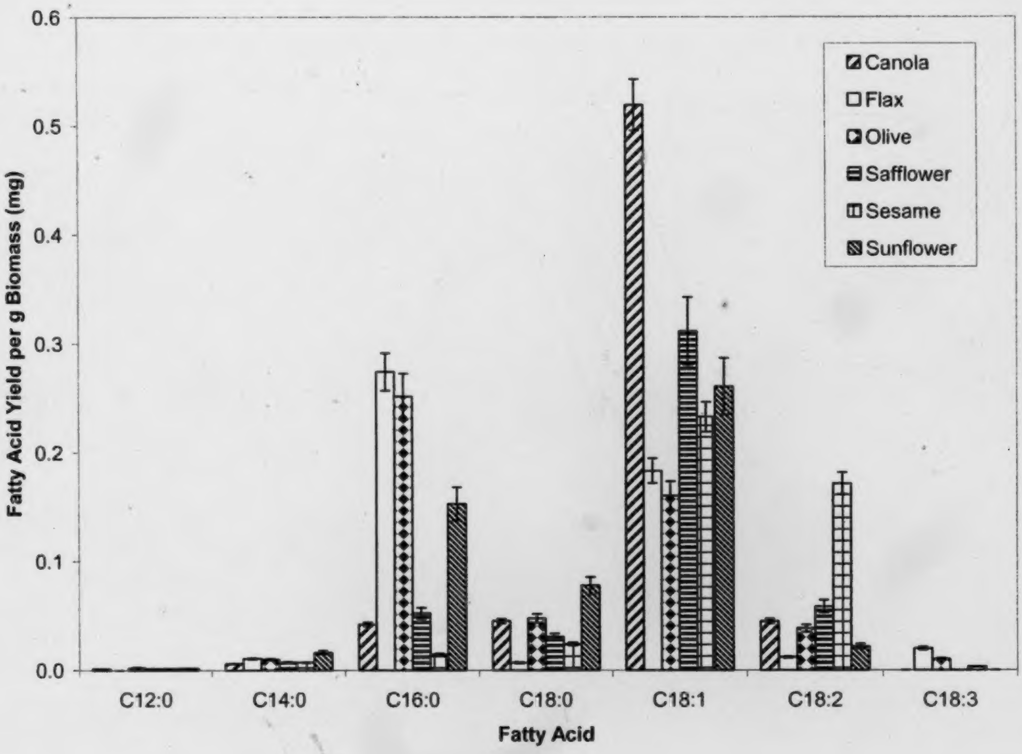

Figure 3-9: Absolute yields of fatty acids per g biomass of media supplemented with various oils.

may be a greater advantage to adding Mn^{2+} metal ions to the fungal base medium at a concentration of 5 mg/L.

The absolute GLA yield obtained using this Mn^{2+} enhanced medium was almost three times of that observed using the basal medium alone (0.0656 g/g biomass and 0.0215 g/g biomass, respectively). The lipid yield was 54.2% of the total dry biomass and the total unsaturated fatty acid content amounted to 84.3% of the total lipid content.

GLA made up 15.8% of the total unsaturated fatty acid yield (14.3% of the total fatty acid yield) which amounts to 13.3% of the total lipid yield. Evening primrose seed typically consists of 18 – 20% oil with a GLA content of 8 - 12% (2). With regards to fatty acid yield, GLA typically makes up 3.00 – 15.00% of the total fatty acid content of evening primrose oil. These results indicate that, under these conditions, this strain of *M. ramanianna* var. *ramanianna* has a slightly higher lipid yield with a slightly higher GLA content than evening primrose seed. In addition, the GLA yield per total fatty acid content is at the high end of that typically obtained with evening primrose oil. In the context of fungal sources of GLA, Table 2-2 shows that there are other fungal strains (including other *M. ramanniana* strains) that give higher yields per total fatty acid content (up to 31.4%). However, the per g biomass and per g lipid yields (i.e., absolute values) were not reported in these studies.

Therefore, the series of experiments that have been performed to date on this locally isolated *M. ramanniana* var. *ramanniana* strain indicate that, for maximum GLA production in this organism, the basal growth medium would consist of 5% dextrose and 1% yeast extract, supplemented with 5 mg/L Mn^{2+} ions and incubated at room temperature (22.4 °C). The results indicate that GLA production in this particular fungal strain offers a viable alternative to industrial GLA production since, under these specific conditions, the GLA yield is at least comparable to those for evening primrose oil (Table 1-1). However, when compared to other fungal strains (as percent total fatty acid yield), including some belonging to the genus *Mortierella*, this particular strain is an intermediate producer of GLA (Table 2-2).

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Chapter 4

General Discussion and Conclusions

Heightened consumer awareness of the importance of essential fatty acids (EFAs) in a healthy diet has resulted in an increase in consumer demand for products containing these nutrients. This increased demand, in turn, has prompted some researchers to seek out alternate sources of EFAs that are relatively high in EFAs, which are relatively inexpensive to produce on an industrial scale. In addition to various plant sources, fungi have become a target for potential commercial production of various EFAs. One fungal genus that has garnered significant research interest in this area is the genus *Mortierella*. The fact that one of these fungal species, *M. alpina*, has been recently employed as a commercial producer of arachidonic acid (ARA) offers validation to the idea that these fungi can be utilized for mass EFA production.

This MSc. project focused on the systematic optimization of several growth variables for the accumulation of *gamma*-linolenic acid (GLA) in a locally isolated (Perryvale, Alberta) *Mortierella ramanniana* var. *ramanniana* strain. The variables tested were pH, temperature, carbon source, carbon concentration, nitrogen source, nitrogen concentration, addition of various metal ions to the basal growth medium, metal ion concentration, and supplementation of the growth medium with various seed oils. Though these variables have been tested for various species of the *Mortierella* genus, most of the focus has been on the species *M. alpina*; few studies have been performed using the *M. ramanniana* species. Furthermore, the experiments that were performed using *M. ramanniana* as the test organism did not focus on systematically optimizing all of the aforementioned variables in such a way that each successive variable depends on the results obtained for the one before it.

Two main series of experiments were performed. Series 1, results of which are summarized in Chapter 2, focused on the effect of pH, temperature, and carbon source on growth, lipid accumulation, and GLA production. Series 2, results of which are summarized in Chapter 3, focused on the effect of nitrogen source, the addition of metal ions, and supplementation of oils to the growth media on biomass, lipid, and GLA yields.

Series 1: Experiment 1 was performed to determine the range of pH's in which this particular *M. ramanniana* strain could grow. Three media were used in this experiment in order to determine whether any media-pH interactions may occur. The results from this experiment showed that this fungus is relatively pH insensitive between the pH's of 3 and 10, regardless of the media used. However, results also indicated that there is some media-pH interaction that occurs. Though the exact mechanism of this interaction is not clear, it most likely involves the hindrance of the mechanism of action of enzymes vital to the growth and propagation of this fungal strain, since enzymes require specific pH conditions under which to properly and efficiently function. The one thing that PDB contained that the other two media tested did not was potato starch. It is possible that this complex saccharide provided an advantage (over the other media tested) to the fungus at the higher pH which resulted in growth at pH 11. Furthermore, the growth trends observed were shown to be attributed to the pH of the media and not to the addition of the compounds that were added to adjust the pH. This was confirmed by growing additional, randomly selected samples which were adjusted using tartaric acid and potassium hydroxide solutions in place of those used to adjust the regular experimental samples.

The large pH range in which this fungal strain can grow is interesting to note when compared to the results obtained from pH experiments performed on other *Mortierella* strains. An experiment by Lindberg and Molin (1) found that *M. alpina* did not grow at pH 8.5 and that there was a decrease in biomasses obtained at pH 7.5 when compared to pH's 5.5. and 6.5. An experiment by Xian *et al.* (2) found that there was an inverse relationship between biomass and pH for *M. isabellina* between pH 5 and 9. Though neither of these studies examined the effect that the full range of pH's had on their test organisms, it is interesting to note that the species belonging to the subgenus *Micromucor* (i.e., *M. isabellina*) grew at a higher pH than the one belonging to subgenus *Mortierella* (i.e., *M. alpina*). *M. ramanniana* is a member of the subgenus *Micromucor*, however, further studies would have to be performed to determined whether there is a difference in the pH range of growth between the two *Mortierella* subgenera.

Series 1: Experiment 2 was performed to determine the range of temperatures that this organism could grow. The same three media that were used in Series 1: Experiment 1 were again used to determine whether any temperature-media interactions occurred. In addition, three pH's (3, 6, and 9) were used for each medium at each temperature tested to determine whether any pH-temperature interactions may occur. The most evident pH-media-temperature interaction was observed at 10 °C where no apparent growth occurred in the CMB media though there was growth observed for the other two media. Furthermore, all three of the media tested showed variations in biomass and lipid yields across pH's when temperature was held constant and across temperatures when pH was held constant. Once again, these variations could most likely be explained by the effective pH and temperature ranges in which fungal enzymes vital to growth and lipid accumulation can work.

A study by Leman and Brakoniecka-Sikorska (3) found that the highest biomass and lipid yields for a *M. ramanniana* var. *angulispora* strain were obtained at a pH of 4.5 and a temperature 30 °C while another study (4) found that the optimal growth temperature for two *M. ramanniana* strains was 25 °C. The same study (4) found that the optimum temperature for GLA production in these fungi was 20 °C. Logic would suggest that there would be an increased level of GLA production in these organisms at lower temperatures in order to help maintain membrane fluidity. Membrane theory dictates that, as temperature decreases, organisms tend to produce and incorporate more unsaturated fatty acids into their membrane bilayers because the presence of double bonds in these fatty acids increases the membrane fluidity. As such, one would hypothesize that maximum GLA production would occur when *M. ramanniana* strains are grown at lower temperatures.

Hansson and Dostalek (4) found that the maximum relative yield of GLA (i.e., percent total fatty acid) was obtained at the lowest temperature tested though optimum growth occurred at 25 °C. The absolute GLA content (g/ g biomass) was not reported. It is possible that, though the relative GLA yield was higher at 20 °C, the absolute GLA yield may actually have been higher at 25 °C since the biomass was maximized. In this current MSc. Project, the fatty acid profiles were not determined during the temperature experiment (Series 1: Experiment 2), however, biomass and lipid yields were recorded.

The reasoning behind this decision was that maximizing lipid yield would ultimately result in maximized GLA yield.

Based on the first two experiments in Series 1 of this current project, it was determined that the locally isolated fungal strain used in this study could be induced to accumulate a considerable quantity of GLA when grown at room temperature and without adjustment of the growth media. Since the goal of these experiments is to determine what conditions may be suitable for the eventual scale-up of GLA production utilizing this fungal strain, the cost of production should be weighed at each stage of these experimental procedures. The results from the first two Series 1 experiments are promising in this regard. Since the pH of the fungal growth medium does not need to be adjusted, this eliminates the cost required for chemicals, above and beyond those required for the basal growth medium. Also, the ability to optimize GLA production at room temperature reduces the energy cost associated with temperature controlling the growth medium. As such, the remaining experiments were performed at room temperature and at the unadjusted pH of the media.

The third set of experiments in Series 1 focused on the effect that carbon source and carbon concentration had on biomass, lipid, and GLA yields. A single nitrogen source (yeast extract) set at a concentration of 1% (w/v) was used for broths containing all carbon sources except PDB which was a complete medium in and of itself. Another important point to note is that PDB, unlike the other media used, contained starch. The other carbon sources tested were simple sugars, with the exception of beet pulp and sodium acetate which is an inorganic carbon source.

The results from this set of experiments showed that beet pulp and sodium acetate were the poorest carbon sources for this fungus while dextrose proved to be the best carbon source with a maximum GLA yield at 5%. FYB and DYB yielded similar biomass results at all concentrations; findings that are consistent with those obtained by Hansson and Dostalek (4).

It is interesting to note that, though beet pulp has been found to be a valuable growth medium for other fungi, this particular *Mortierella* strain did not effectively utilize the beet pulp-containing media. The preferential and effective utilization of simple sugars by these fungi is not surprising given the fact that they are saprophytic, *r*-selection organisms (5, 6). Organisms that follow the *r*-selection lifecycle are usually the first to populate an area, exploit any readily available food source then rapidly reproduce before dying off. For this reason these species are often referred to as pioneer organisms. Given the fact that *Mortierella* spp. are pioneer fungi, it makes sense that they would be adapted to the exploitation of simple sugars in lieu of more complex molecules. Simple sugars are more desirable as consumables due to the fact that they require less metabolic modification by an organism before they can be efficiently utilized. This means that an organism does not have to expend as much energy breaking down the molecules to a more usable form. In the case of *Mortierella* spp., this would be especially valuable since they are adapted to consumption of nutrients that facilitate rapid proliferation.

It is, however, unfortunate that the beet pulp media did not yield more promising results. Beet pulp is a low-value by-product of sugar manufacturing and, as such, would have offered a low cost, readily available fungal growth medium for the industrial fungal production of GLA. However, since most of the simple sugars have been removed by the manufacturing process, it is not surprising that the beet pulp itself was a poor carbon source for this fungal strain.

Upon completion of the first series of experiments, results indicated that this fungal strain yielded GLA contents that were in the range of those reported for other *Mortierella ramannniana* strains. The GLA produced comprised 11.6% of the unsaturated fatty acid yield (8.3% of the total fatty acid yield) which amounts to 8.2% of the total lipid yield. Evening primrose oil, which is the major industrial source of GLA, typically yields 3.00% - 15.00% of its total fatty acid content as GLA. This indicates that, if the production costs can be kept below that which is required for evening primrose GLA production, this fungal strain could be a viable alternative for industrial GLA production. However, there are other fungal strains, including other those in the *M. ramanniana* var. *ramanniana* species, that have GLA yields of up to 31.4 % of their fatty acid contents.

Series 2: Experiment 1 focused on the manipulation of nitrogen source and nitrogen concentration and the effect that this had on biomass, lipid, and GLA yields. A set concentration of dextrose (5%) was used as the carbon source for all of the nitrogen conditions tested. This decision was based on the results from Series 1: Experiment 3 where GLA production was maximized with 5% dextrose when the nitrogen concentration was set to 1%. Results indicated that the best nitrogen source for biomass and lipid yields in this *M. ramanniana* strain was yeast extract at a concentration of 0.5% when dextrose was set at a concentration of 5%. However, the highest GLA yield was obtained with a yeast extract concentration of 0.25%. These findings are consistent with those of other studies where yeast extract was tested as a nitrogen source for various

Mortierella strains (9 –11). In each case, yeast extract at the lowest concentration tested (1%) was the best nitrogen source for all three of these variables when the carbon source was set at either 2% or 4%. Unlike the other nitrogen sources tested, yeast extract contains all of the essential nutrients (including phosphorous and various metal ions) required by most organisms for growth. As such, it is not surprising that it is often the best nitrogen source for the growth of most fungal strains. The second best nitrogen source tested in this project was peptone. Although peptone does contain many of the nutrients required for the growth of most fungi, it does not contain all of them. As such, it is an intermediate nitrogen source when compared to the other three nitrogen sources tested in this project. Both casein and *L*-lysine primarily contain only nitrogen as an essential element which explains why they are unable to support substantial fungal growth and are usually used in complex media which include metal ions and other essential elements.

These results are consistent with the theory that limiting nitrogen in the presence of excess carbon leads to the cessation of proliferation and triggers lipid accumulation (6). However, when determining which nitrogen concentration is optimal for GLA production, it is important to take into account both the production of GLA on a per g biomass basis and the absolute GLA content. The absolute GLA content is the total amount of GLA accumulated for the whole fungal biomass. A given growth condition may yield high per g biomass GLA contents with low biomass yields while another set of conditions may yield low per g biomass GLA content with high biomass yields. The resulting absolute biomass may be similar in both conditions but the GLA production is more efficient in the condition that yielded high amounts of GLA on a per g biomass basis. When comparing the two growth conditions, the same amount of resulting biomass would contain higher GLA yields under the first set of conditions when compared to the second.

Based on the results from the previous experiment, the remaining experiments were performed using a basal medium consisting of 0.25 % yeast extract and 5% dextrose since the highest GLA content was obtained using this media composition. Series 2: Experiment 2 was performed to determine the affect that the addition of various metal ions to the basal medium at different concentrations had on biomass, lipid, and GLA yields. Series 2: Experiment 3 focused on the affect that supplementation of the basal medium with various seed oils had on these three variables.

The results from the metal ion experiments indicated that different metal ions at different concentrations do have a significant impact on the biomass yield, lipid accumulation, and fatty acid profile of this fungal strain. The addition of Cu^{2+}, Mg^{2+}, or Zn^{2+} to the basal medium outlined above showed an inverse relationship between biomass and metal ion concentration while a direct relationship was observed between these two variables with the addition of Fe^{2+}. No trend was observed in the relationship between biomass and metal ion concentration for the Ca^{2+} and Mn^{2+} and none of the biomasses observed for any of these metal ion supplemented media exceeded those obtained with the basal medium alone. Similarly, various trends were observed for lipid yields and fatty acid profiles in this fungus, depending on which metal ion was added to the basal medium.

Previous studies have looked at the effect of various metal ions and metal ion concentration on biomass, lipid, and target fatty acid yields and it is also clear from those studies that various metal ions have different effects on all three of these variables (4, 10). However, a study performed on two different strains of *M. ramanniana* yielded contrary results from those obtained in this Masters project. Hansson and Dostalek (4) found that Cu^{2+} and Zn^{2+} had positive effects on lipid accumulation and GLA production while the results from this current study indicated that the addition of Cu^{2+} to the basal medium actually had a negative affect on GLA production in the strain of *M. ramanniana* tested in this experiment. In fact, the GLA contents recorded for Cu^{2+} supplemented media were among the lowest values obtained for this set of experiments. However, there was increased accumulation of GLA in media containing either Zn or Mn metal ions over and above that which was produced by fungal growth in the basal medium itself.

The importance of metal ions in biological systems is related to their roles as enzyme cofactors. As is the case with temperature and pH, the presence of particular metal ions must be optimized for maximum enzyme performance and the specific requirements for these optimizations vary with enzymes. The results from the studies that have investigated the effect that different metal ions have on *Mortierella* growth, lipid accumulation, and fatty acid composition (including this project) seem to indicate that the specific metal ion requirements vary within the genus. This would suggest that, though similar processes are being carried out within each fungal strain (i.e., proliferation, lipid accumulation, fatty acid production) the specific enzymes that are involved in these processes vary from strain to strain.

The results from Series 2: Experiment 3 indicate that supplementation of the basal growth medium with any of the seed oils tested in this study does not offer any advantage over and above those yields obtained using the unsupplemented basal medium. In fact, there was a general decrease in the amount of GLA produced by the seed oil supplemented media when compared to the unsupplemented basal medium itself. Previous studies that have investigated the affects of supplementation of fungal growth media with oils focused on *Mortierella* fungi that can produce C20 PUFAs with arachidonic acid (ARA), dihommogamma-linolenic acid (DHGLA) and eicosapentaenoic acid (EPA) being the main target fatty acids. The immediate precursor for DHGLA is LA and DHGLA itself is the immediate precursor to ARA. Research has shown that the enzyme responsible for the conversion of DHGLA to ARA (*delta*5-desaturase) is inhibited by the presence of sesame oil in the growth media (11-14). A number of studies have also been performed using linseed (flax) oil (15-17) which contains a large proportion of *alpha*-linolenic acid, a precursor of EPA.

Since the oil offers lipid components that can be incorporated into and modified by the fungus, it is logical to assume that the fungus would preferentially utilize these readily available components rather than synthesizing them *de nova* from the carbon source provided. Therefore, following this rationale, it would be logical to assume that the fatty acid composition of the oil that is used to supplement the basal medium would affect the resulting fatty acid profile of the fungal oil. The results from this experiment appear to support this assumption. However, the goal of using oils to supplement the basal medium was to test whether the presence of LA (the immediate GLA precursor) in the oils would increase GLA production in this fungal strain. In this instance, oil supplementation generally hindered GLA accumulation in this fungus. The one exception to this was flax oil which accumulated a similar per g biomass GLA content to that of the basal medium.

The series of experiments that have been performed for this MSc. thesis indicate that the optimum growth conditions for GLA production in this locally isolated *M. ramanniana* var. *ramanniana* strain would consist of a basal growth medium containing 5% dextrose and 0.25% yeast extract supplemented with 5 mg/L of Mn ions and incubated at room temperature (22.4 °C).

Though the addition of Mn^{2+} at a concentration of 5 mg/L to the basal growth medium almost tripled the GLA yield per g biomass (from 0.0215 g/g biomass to 0.0656 g/g biomass), its unsaturated fatty acid content only consisted of 15.8% GLA which amounted to 13.3% of its total lipid yield. This is only a 4.2% and 3.1% (respectively) increase in GLA production relative to lipid production. This still indicates that this strain could be a potential industrial producer of GLA, however, there are other fungal strains that could prove to be more efficient industrial GLA producers (Table 2-2) on a per total fatty acid yield basis.

Despite these results, it is important to do systematic testing on novel fungal strains (even within a known species) to determine the optimum strain for EFA production. For example, a number of studies looked ARA production in various strains of *M. alpina* before the 1S-4 strain became the favourite experimental specimen among the species. Likewise, it is important to research new *M. ramanniana* strains to determine their viability as potential GLA producers.

Further experiments could be performed to determine whether the GLA production of this particular fungal strain could be further maximized. These studies could include different variables within the same factors studied in the above six experiments or they could include more novel factors. For example, the same carbon, nitrogen, metal ion, and oil supplementation sources could be further investigated by testing concentrations of these variables other than those cited in this project and different sources within these factors could also be tested. Also, any potential interactions between these variables could be investigated by varying more than one variable at a time. Some potential interactions that may be of interest include carbon-nitrogen, metal ion-oil supplementation, and any other combination of these four variables. Further studies could also be done involving pH and temperature variables. In addition, novel factors such as aging could be investigated both independently and in conjunction with other factors. In this project, the cultures were all allowed to grow for ten days prior to harvesting the mycelia. Future research with this organism could involve harvesting mycelia at different days of growth prior to immediate lipid extraction as well as harvesting mycelia of different ages and allowing them to stand for a number of days prior to lipid extraction. The objective of all the these proposed studies would be to balance biomass, lipid, and GLA yields such that the absolute GLA yield is maximized.

Even if the GLA content of this fungus was optimized above other fungal strains, further studies would still need to be performed to determine the optimum conditions for scale-up as previous studies have shown that there are special considerations that must be made for this to happen (e.g., aeration of large vat cultures). However, the current utilization of *M. alpina* as an industrial source of ARA lends credibility to the idea that *M. ramanniana* may be a viable option for eventual industrial production of GLA.

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